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The Journal of LABORATORY and CLINICAL MEDICINE

VOL. 49 JANUARY, 1957 NO. 1

The Official Publication of the Central Society for Clinical Research

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The Official Publication of the Central Society for Clinical Research

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LETTER FROM THE EDITORS

The following members of the Board of Editors have accepted reappointment for a second three-year term: Dr. Frank H. Bethell, Dr. Elmer L. DeGowan, Dr. Richard E. Ebert, and Dr. Richard L. Landau.

During the present year the Editors and Board Members have called upon their associates and colleagues in other universities to aid them in the evaluation of manuscripts. Those who have participated in this capacity during the past year are:

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We wish to take this opportunity to thank the members of the Editorial Board for their present and continuing service to the JOURNAL and to the Central Society for Clinical Research. We also wish to express our appreciation for the cooperation received from The C. V. Mosby Company.

WILLIAM D. ROBINSON, M.D., Editor
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THE ESSENTIALITY OF CLINICAL RESEARCH: SOME COMMENTS ON ITS ROLE IN INSTITUTIONS WHICH PRACTICE AND TEACH MEDICINE*

RANDALL G. SPRAGUE, M.D.
ROCHESTER, MINN.

SERVING as president of the Society during the past year has been a privilege which I have prized. At this time I would like to express my gratitude to our capable secretary-treasurer, Dr. Robert H. Ebert, whose efforts have made my task a light one, and to the Council for its conscientious handling of the affairs of the Society. I shall avail myself of this opportunity for self-expression which you grant each year to your president by making some comments on the essentiality of clinical research in the life and work of institutions which practice and teach medicine.

We are living in the golden age of medical discovery. It has been said that medicine advanced more in the first fifty years of the twentieth century than in all preceding time. The pace of progress is still accelerating. Great discoveries in fundamental fields—antibacterial agents, hormones, vaccines, radioactive isotopes, pharmacologic agents, and many others—have stirred the imaginations of teams of clinical investigators and basic scientists working together on problems of human disease. Medicine literally has been made over.

THE GENERAL PROBLEM

In this astonishing advance of medicine, clinical investigation, in concert with practice and teaching, has played a vital role. Concurrently, it has become an essential ingredient in the life and work of institutions in the forefront of medical practice and teaching. And yet, possibilities of encroachment on the scholarly activities of medical institutions are ever present, and it is appropriate periodically to call attention to the importance of keeping clinical research in proper relationship to care of patients and teaching. There is no denying that clinical investigation has been on the rise since the war, but so also have other demands on the resources for research, particularly the human resources.

Almost everyone is in favor of research, at least in the abstract sense. Is not research the principal means of medicine's attack on unsolved riddles of prevention and control of illness, alleviation of suffering, and saving of human lives? Is it not the ultimate means of solution of many of the vexing

* Presidential address given at the Twenty-ninth Annual Meeting of the Central Society for Clinical Research, Chicago, Ill., Nov. 9 and 10, 1956.

From the Section of Medicine, Mayo Clinic and Mayo Foundation, Rochester, Minn. The Mayo Foundation is a part of the Graduate School of the University of Minnesota.

problems of insuring good medical care for the population? Indeed, to oppose research today would be like opposing virtue. This passive approval of research, unencumbered by responsibility for its implementation, pervades many institutions but accomplishes little. The real necessity is the activation of clinical research by clinicians through provision of time, personnel, and funds. Availability of funds from government and other sources to support research has not entirely solved the problem, and cannot, for it is basically one of the philosophy of medicine in each institution. Those who determine policy and action in such matters have a most difficult task, for they must adjudicate while impaled on multiple horns of multiple dilemmas arising from the practical problems of making ends meet financially, maintaining teaching programs, and meeting the needs of patients for prompt, thorough care. In spite of sympathetic recognition of the administrative difficulties, one cannot escape the conclusion that in this contested apportionment of time and funds, research too often takes the hindmost. If medical institutions are to fulfill their highest obligations, they must not orient themselves toward practicalities only and permit ideals to yield to material pressures.

The teaching hospitals and clinics with research programs constitute only a small fraction of all the medical institutions of our country; nevertheless, a much greater number of hospitals and clinics have assumed responsibility for teaching, in addition to practice, through their internship, residency, and postgraduate training programs. A recent comment, quoted in the American Foundation's midcentury survey of medical research, puts it that "modern hospitals are medical teaching centers, good or bad, whether they wish it or not."[1] Thus, if there is validity in the thesis that clinical research is an essential ingredient in institutions which aspire to the best in care of patients and in teaching, there still remains a broad field for expansion of clinical investigation in this country.

SIGNIFICANCE IN THE TRAINING OF YOUNG MEN

Research in the basic sciences is fundamental to medical progress, but it is the inquisitive physician who must bridge the gap between the basic sciences and the patient in the ward. To this end, a spirit of inquiry must be cultivated in a segment of the profession from which will emerge the future leaders of medicine. Most of these will come from among the large numbers of graduates of our medical schools who aspire to be specialists. While few of these will develop into medical scholars in the high sense which this Society represents, nevertheless their best possibilities for leadership in medicine will be realized if during their postgraduate training they are exposed not only to many patients but also to the scholarly discipline of clinical investigation. Out of this distillation will come the few giants of the medical science of the future. This premise was well expressed 39 years ago by Dean Lyon of the University of Minnesota Medical School in a proposition ". . . that the leaders of medicine, as exemplified by the class of specialists, should be more than practitioners, they should be scientists; that leadership in the profession should rest on productive scholarship . . ."[2]

A seemingly contrary view of that part in the training of specialists which should be devoted to scientific research is suggested in a question asked by Lewis: "Does it really seem profitable or logical for a young man, who does not have that interest and that aptitude, to spend a year where he functions intellectually not far above the level of a technician?"[3]

These apparently discrepant views can be reconciled if one accepts a broad definition of clinical research, which does not limit the term to laboratory observation utilizing the most technical methodologies of this technicologic age. The important aspect then seems to be to develop in the young physician the capacity and discipline to collect, integrate and critically analyze sound data from the clinic, the laboratory, and the literature pertinent to unanswered clinical problems. In this broad sense, scholarly activity of some sort, in contrast to what might be likened to training for a craft or trade, should be a part of the education of every young physician who aspires to specialization, and a part of the life in every institution which participates in medical education at any level. Again quoting Dean Lyon, ". . . in all places where clinical graduate work is undertaken, the student must be held back from clinical plethora, from engorgement with routine observation, diagnosis and care of the sick. He must be given time for laboratory and library work; for meditation and expansion; for the development of originality. . . . If we don't do this, the research aspects of our work will dwindle away. Our product will be trained and safe, but sterile. Our graduate medical schools . . . will be amplified, but not glorified, polyclinics."[2]

ESSENTIALITY TO THE INSTITUTION AND ITS STAFF

Carl Linnaeus pointed out one path to scientific honor and glory when he said, "A professor can never better distinguish himself in his work than by encouraging a clever pupil, for the true discoverers are among them, as comets amongst the stars." Likewise, the prestige of a medical institution is closely linked to that of its staff, and thus is dependent on the provision of opportunities for the young staff member to make his mark in medicine. Eventually his scholarly attainments, or lack of them, are reflected in his own reputation and that of his institution. The eminence and distinction of both are by-products of his professional growth with respect to ability not only to care for patients, but also to contribute to the progress of medicine through study of disease.

Scholarly achievement in medicine is difficult when little or no time is specifically allocated for research, and when the waking hours are subject to the demands of an excessive, unpredictable, or variable clinical practice which for economic or humanitarian reasons must be assigned the highest priority. Under such circumstances the young man's accomplishments in investigation are made at the cost of unwarranted sacrifice of self and family. Human energy is exhaustible. Although occasionally great experiments have been conceived in the deep of night, this is not generally the most favorable time for creative

thinking. The frustrations associated with trying to do investigative work without specific allocation of time for this purpose inevitably have a demoralizing effect which is in itself destructive to creative research. To have ideas without time to pursue them is stultifying; eventually the flow of ideas stops.

By contrast, the satisfaction to be derived from an orderly program of clinical investigation lends flavor, tone and spirit to the life in an institution. The joy that comes from medical discovery and its application in the care of the ill is not duplicated, I am sure, in either research or practice by themselves, nor, indeed, in any other calling of man.

Obviously, in the formulation of policies concerning allocation of staff time for care of patients, for teaching, and for research, not to mention administration, careful thought must be given to what is possible in the local setting as well as to what is desirable. These functions may be interrelated economically as well as intellectually, and humanitarian considerations in the care of patients are very persuasive. The balance among them will vary in different institutions, but regardless of economic and other pressure all three must permeate the life and thinking in any institution which would serve the noblest aspirations of medicine.

MAINTENANCE OF HIGH STANDARDS OF PATIENT CARE

In the last analysis, hospitals and clinics are dedicated to the care of the sick through skillful application of advancing knowledge of disease. This high duty will be performed best if through research and teaching there is progressive improvement in medical practice so that the benefits of science are made available to all patients. The ancient statement of Plutarch has been applicable to medical progress through the ages: "Medicine, to produce health, has to examine disease," just as "music, to create harmony, must investigate discord." The finest standards of medical practice will be maintained in a climate of scientific study of disease, where physicians are continuously concerned with the distinction between fact and appearance in human ailments, until empiricism is replaced by knowledge.

At a more elementary level of study of disease, astute physicians recognize that for the benefit of patients, time is needed for study and periodic review of procedures employed in diagnosis and treatment. How often do we physicians persist in the use of diagnostic and therapeutic methods which have become a part of the folklore of our institutions simply because we lack the time to look at them critically and thereby recognize their deficiencies? As a minimal requirement, a hospital or clinic should have a simple program of study aimed at critical review of diagnostic and therapeutic methods together with statistical studies of at least local interest. Then the institution will bear no unsavory resemblance to a workshop for the plying of a trade. Orderly exploitation of even simple resources for research under proper auspices can stimulate a desirable critical attitude toward clinical problems which will be reflected in a better quality of medical practice.

MORAL OBLIGATION TO CONTRIBUTE TO MEDICAL PROGRESS

A moral obligation to contribute to medical progress inheres in the activities of caring for patients and teaching students. The institution which functions as a purveyor of medical care, albeit by high standards, but fails to create better medicine through research and study, fails to discharge its moral obligation. New knowledge rarely comes without study. If the experimental investigation of pernicious anemia had ceased with Minot and Murphy's classic description in 1926 of the beneficial effects of a diet rich in liver, understanding of the disease would still be in a relatively primitive state. Likewise, the mere administration of kilograms of adrenal cortical hormones to thousands of patients is not likely to elucidate the mechanism and real significance of the anti-inflammatory effects. This will be achieved by continued meticulous study, not by indiscriminate exhibition of the drugs. Thus, fulfillment of the moral obligation to contribute to medical progress depends on creative scholarship in some form. Neither the provision of good care to large numbers of patients by established methods nor the teaching of what is known without adding some increment to it discharges the responsibility. As William Bean[4] pointed out from this platform five years ago in another connection, to focus attention on a "backward view of medicine or at best the status quo" is an evidence of moral irresponsibility in medicine.

SUMMARY

And so permit me to summarize and conclude. In the life and work of medical institutions clinical research has an essential importance which cannot be disregarded if the goal is something better than the ordinary in practice and teaching. An atmosphere of scholarly inquiry into the mechanisms of disease, and means of their correction, fosters better training of young men, maintenance of institutional prestige, satisfying achievement by the staff, improved medical care for patients and, finally, a gratifying fulfillment of the moral responsibility to contribute to the progress of medical science. May our members in their respective institutions continuously serve the fine purpose of our Society—the cultivation of clinical research and the development of younger men! May they not merely apply and teach an evolving medicine, but be forces in that evolution!

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CLINICAL AND EXPERIMENTAL

EFFECT OF CORTISONE ON THE ABNORMAL DISTRIBUTION OF INTRAVASCULAR WATER IN ADRENAL CORTICAL INSUFFICIENCY IN MAN

JOSEPH F. DINGMAN, M.D.,* DAVID H. P. STREETEN, M.B., D.PHIL.,** AND GEORGE W. THORN, M.D.
BOSTON, MASS.

THE well-known inability of patients with adrenal insufficiency to excrete administered water forms the basis of a useful clinical screening test described by Robinson, Power, and Kepler.[1] This procedure, however, yields no information as to possible defects in the distribution of body water which may predispose to faulty renal excretion. A study of the distribution of the administered water throughout the total body water should yield additional information regarding this abnormality in water excretion. However, methods currently available for investigating changes in body water distribution may be associated with errors large enough to mask the alterations in water distribution which occur following the administration of 20 ml. of water per kilogram of body weight (equal to approximately 3 per cent of the total body water).

The present report describes studies of the distribution of intravascular water between the plasma and erythrocytes as calculated from changes in hematocrit and hemoglobin measurements during standard water tests performed in patients with adrenal cortical insufficiency. An abnormal increase in erythrocyte volume which was not associated with significant abnormalities in the hydration of the plasma accompanied the delayed water diuresis observed in untreated patients. The restoration of a normal diuretic response to water administration with cortisone therapy was associated with a return of the erythrocyte response to normal.

MATERIALS AND METHODS

Robinson, Power, and Kepler tests of water excretion[1] were performed on normal subjects, patients with Addison's disease, and bilaterally adrenalectomized subjects. The method of study included hourly measurements of serum base,[2] serum total protein,[3] urinary specific gravity (with use of a standard clinical urinometer), and water excretion for a four-hour period following the oral administration of 20 ml. water per kilogram of body weight. Heparinized venous blood specimens (about 1 mg. heparin powder per 8 ml. blood) were

From the Department of Medicine, Harvard Medical School and the Peter Bent Brigham Hospital, Boston, Mass.

These studies were carried out with the aid of grants from the National Institutes of Health, United States Public Health Service; the Eugene Higgins Trust of the Harvard Medical School; the Armour Laboratories; Merck and Company; The Upjohn Company; and Ciba Pharmaceutical Products.

Presented in part at the Endocrine Society Meeting, Chicago, Ill., June, 1952.

Received for publication May 31, 1956.

*This work was done during the tenure of a fellowship from the Dazian Foundation for Medical Research. Present address: Department of Medicine, Tulane University School of Medicine, New Orleans, La.

**Present address: Department of Internal Medicine, University of Michigan Hospital, Ann Arbor, Mich.

obtained prior to hydration and hourly thereafter. Hematocrits were measured in duplicate in closely matched Wintrobe tubes, according to standardized techniques.[4] The tubes were spun at 3,000 r.p.m. for 30 minutes in an International centrifuge (centrifugal force 1,035 g). The blood hemoglobin concentration was measured on 1:500 dilutions of whole blood in dilute ammonia solution at 540 mμ in an Evelyn spectrophotometer calibrated with blood samples of known oxygen capacity.

In some of the normal subjects and patients with Addison's disease in whom water tests were performed, an additional quantity of blood was obtained prior to hydration for simultaneous study of the response of the erythrocytes to hemodilution in vitro. One milliliter of whole blood was placed in each of four 5 ml. test tubes, tube 1 serving as the control. Depending on the initial hematocrit (Hct.), a volume of distilled water measured with a micropipette was added to the last 3 tubes to produce dilution of the plasma to a calculated tonicity of 0.85, 0.80, and 0.70 per cent saline solution, respectively, assuming 0.90 per cent to represent the tonicity of undiluted plasma. This procedure produced a percentile dilution of the whole blood over a wide range which exceeded that observed in vivo* (Fig. 2). The tubes were stoppered, mixed, and gently agitated in a rotatory test tube rack at 37° C. for one hour. The Hct. of each blood specimen was then measured in duplicate in closely matched Wintrobe tubes, and the hemoglobin (Hgb.) concentration of the undiluted blood was measured in duplicate. In three of the in vitro studies on patients with untreated Addison's disease, 0.5 mg. crystalline hydrocortisone† was added to a second group of four tubes. These tubes were then carried through the above procedure simultaneously with the tubes to which no hormone was added.

CALCULATIONS

The formula of Elkinton, Danowski, and Winkler[5] for calculation of changes in plasma volume (P. V.) based on Hct. and Hgb. determinations has been extensively applied to study alterations in hydration of the plasma. This formula "rests on the assumption that no erythrocytes have been removed from or added to the circulating blood and that the Hct. and Hgb. represent the average of these values throughout the circulation." The subscripts 1 and 2 refer to the initial and subsequent determinations, respectively.

$$\frac{PV_2}{PV_1} = \frac{1 - Hct._2}{1 - Hct._1} \times \frac{Hgb._1}{Hgb._2} \text{ (I)}$$ [5] Therefore, the percentage change in PV (ΔPV%)

$$= 100 \left(\frac{PV_2}{PV_1} - 1 \right) \text{ (II)}$$

Since the blood Hgb. concentration varies inversely with the degree of hydration of blood,‡ provided the above assumption is correct, the ratio $\frac{Hgb._1}{Hgb._2}$ in this formula estimates the relative change in blood volume (BV) produced by movements of water into and out of the vascular compartment.

Therefore,

$$\frac{BV_2}{BV_1} = \frac{Hgb._1}{Hgb._2} \text{ (III)}$$ and percentage change in BV (ΔBV%) $= 100 \left(\frac{BV_2}{BV_1} - 1 \right)$ (IV)

Since erythrocyte volume (EV) equals blood volume × Hct. then

$$EV_1 = (BV_1)\ (Hct._1) \text{ and } EV_2 = (BV_2)\ (Hct._2)$$

$$\frac{EV_2}{EV_1} = \frac{Hct._2}{Hct._1} \times \frac{BV_2}{BV_1}$$

$$\frac{EV_2}{EV_1} = \frac{Hct._2}{Hct._1} \times \frac{Hgb._1}{Hgb._2} \text{ (V)}$$

and percentage change in EV (ΔEV%) $= 100 \left(\frac{EV_2}{EV_1} - 1 \right)$ (VI)

*This degree of hypotonicity was not accompanied by visible evidence of hemolysis.

†Supplied through the kindness of Dr. H. F. Hailman, The Upjohn Company, Kalamazoo, Mich.

‡This concept is implicit in the spectrophotometric determination of Hgb., which adheres closely to the Lambert-Beer law. This can be demonstrated by measuring the density of serial dilutions of a single specimen of blood of known Hgb. concentration. The density of each solution, when plotted on a linear scale against concentration, will fall on a straight line which intersects the zero point of the graph. The density, therefore is directly proportional to the final Hgb. concentration which, in turn, is inversely related to the dilution of the initial blood specimen.

These formulas may be used to estimate relative changes in EV produced by passage of water into and out of the red cells.* The rationale for the use of formula V is confirmed by the fact that the Hgb. concentration of the erythrocytes has been shown to be inversely related to the water content of the cells.[6]†

$$\frac{M.C.H.C._1}{M.C.H.C_2} = \frac{\frac{Hgb._1}{Hct._1}}{\frac{Hgb._2}{Hct._2}} = \frac{(Hgb._1)(Hct._2)}{(Hct._1)(Hgb._2)} = \frac{EV_2}{EV_1}$$

The percentage change in BV produced by in vitro dilution of blood is equal to the volume of water added to each 1 ml. volume of blood × 100. The Hgb. concentration of the blood in Tubes 2, 3, and 4 was calculated as follows: $Hgb._2 = \frac{BV_1}{BV_2} \times Hgb._1 = \frac{Hgb._1}{BV_2}$

where $BV_1 = 1.00$, and $BV_2 = 1.00$ + ml. water added. In the in vitro studies ΔPV% and ΔEV% were calculated using Formulas I, II, V, and VI (Table II).

The data obtained in this study were analyzed according to accepted statistical methods.[7]

OBSERVATIONS

I. *Tests of Water Excretion (see Table I and Fig. 1).—*

A. Normal subjects: The mean hourly percentage changes in BV, PV, and EV, and the changes in serum total base observed in 13 normal subjects following water administration are listed in Table I, A. Changes in water excretion and urinary specific gravity are shown in Table I, B. Hypotonic expan-

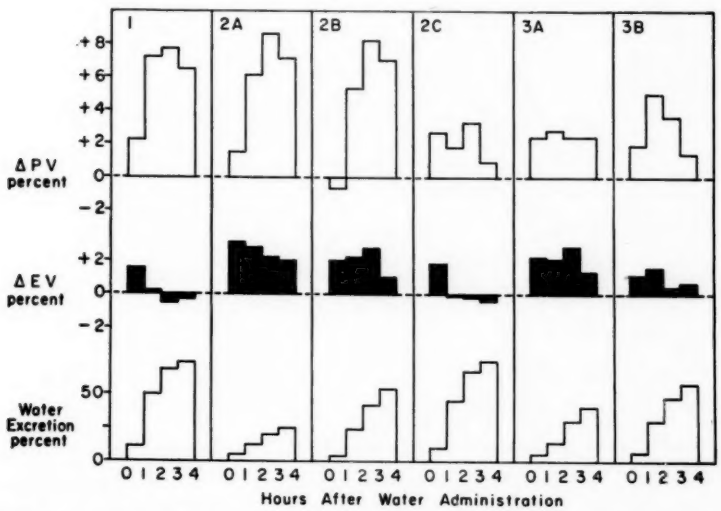

Fig. 1.—Response to water loading in normal, Addisonian, and adrenalectomized subjects. Illustration of mean values for ΔPV per cent, ΔEV per cent, and cumulative water excretion in patients listed in Table I. Numerals above each section refer to corresponding groups in Table I.

sion of plasma volume was observed at the end of the first hour with ΔPV% equal to +2.3 per cent, and the decline in serum base concentration equal to 2 meq. per liter. The increase in plasma hydration was accompanied by a simultaneous increase in erythrocyte hydration with a rise in EV of +1.5 per cent.

*Erythrocyte volume as herein defined should not be confused with the terms "cell volume" or "relative cell volume," which are commonly used to denote the percentage volume of red cells (Hct.) per unit volume of whole blood. EV may increase with the passage of water into the cells but the Hct. may be observed to actually decrease if, with hydration of the blood, PV increased to a greater degree than EV.

†Changes in mean corpuscular hemoglobin concentration (M.C.H.C.) should also be inversely related to alterations in erythrocyte hydration.[4]

TABLE I, A. MEAN RESULTS DURING TESTS OF WATER EXCRETION IN PATIENTS WITH ADDISON'S BEFORE AND AFTER OPERATION, COMPARED TO

GROUP	DIAGNOSIS	NO.	ΔBV%‡ HGB.$_1$*	1†	2	3	4	ΔPV% 1–HCT.$_1$*	1	2	3	4
1.	Normal subjects	13	15.2	1.9	3.9	3.9	3.3	53.7	2.3	7.2	7.7	6.5
	S.E.M.§		0.28	0.62	1.48	0.99	0.84		1.15	2.87	1.64	1.59
2.	Addison's disease											
	A. No cortisone	10	13.3	2.0	4.8	6.0	5.0	59.3	1.5	6.1	8.6	7.1
	S.E.M.		0.51	1.33	1.19	1.34	0.98		2.21	1.74	1.98	1.42
	B. 12.5 to 25 mg. cortisone	6	14.3	0.5	3.9	5.7	4.4	56.7	–0.7	5.3	8.2	7.0
	S.E.M.		0.56	1.39	1.23	1.66	1.90		2.36	2.23	2.46	2.74
	C. 100 to 200 mg. cortisone	9	13.2	2.3	1.0	1.9	0.5	60.2	2.7	1.8	3.3	0.9
	S.E.M.		0.62	1.61	1.76	1.83	1.43		2.62	2.84	2.96	2.32
3.	Adrenalectomy											
	A. Before operation	5	12.9	2.4	2.5	2.6	2.0	60.5	2.5	2.8	2.4	2.4
	S.E.M.		0.46	1.48	2.06	2.46	1.83		2.57	3.35	3.55	2.90
	B. After operation	5	12.9	1.7	3.6	2.3	1.2	59.5	1.9	5.0	3.6	1.5
	S.E.M.		0.41	2.25	1.30	1.52	1.39		3.16	2.23	2.36	1.94
Level of Significance of Differences Between Means ("t" Test)‖												
Groups	1 and 2A		h. s.	—	—	—	—	h. s.	—	—	—	—
	1 and 2B		—	—	—	—	—	—	—	—	—	—
	1 and 2C		—	—	—	—	—	h. s.	—	—	—	—
	1 and 3A		h. s.	—	—	—	—	h. s.	—	—	—	—
	1 and 3B		h. s.	—	—	—	—	h. s.	—	—	—	—

*Initial determinations.

†Hours after water administration.

‡All values for ΔBV, ΔPV, and ΔEV are positive except for those carrying a negative sign.

§Std. error of mean (S.E.M.) $= \sqrt{\frac{\text{S.D.}}{\text{N}}}$

‖h.s. = highly significant $p<0.01$.
s. = significant $p<0.05$.
– = not significant $p>0.05$.

"t" $= \frac{\text{difference between means}}{\text{s.d.}} \sqrt{\frac{N_1 N_2}{N_1 + N_2}}$

Although urinary specific gravity had begun to decline at the end of the first hour, a rise in urine flow had not yet ensued. Maximal diuresis occurred in the second hour and was associated with a return of EV and serum base concentrations to normal. Plasma volume continued to expand, however, and remained elevated throughout the four-hour period, even though the water diuresis had subsided by the fourth hour.

B. *Patients with Addison's disease:*

1. PATIENTS NOT ON CORTISONE THERAPY.* (Table I, *2A*, and Fig. 1):

Five men and five women between the ages of 33 and 63 years were studied. Seven patients had not received desoxycorticosterone acetate (DCA) therapy for at least 2 months prior to study. One patient had received 60 mg. desoxycorticosterone trimethylacetate (DCTMA) intramuscularly one week prior to these

*One patient had been receiving 25 mg. cortisone daily, which was withdrawn for three days prior to study.

DISEASE BEFORE AND AFTER CORTISONE THERAPY, AND IN BILATERALLY ADRENALECTOMIZED MEN
RESULTS OBSERVED IN NORMAL SUBJECTS

ΔEV%					SERUM TOTAL BASE MEG./L.				
HCT.$_1$*	1	2	3	4	0*	1	2	3	4
46.3	1.5	0.2	−0.5	−0.3	149	147	148	149	149
0.84	0.25	0.44	0.23	0.23	0.8	0.8	0.8	0.8	1.1
40.7	3.1	2.8	2.2	2.1	146	142	143	144	141
1.48	0.55	0.94	0.57	0.55	1.5	3.4	0.4	1.5	0.8
43.3	2.0	2.2	2.7	1.0	146	142	143	144	145
1.48	0.38	0.72	1.64	1.52	1.0	2.0	1.5	1.0	1.0
39.8	1.8	−0.1	−0.2	−0.3	145	142	143	144	144
1.68	0.52	0.56	0.57	0.54	0.8	1.1	0.8	1.1	0.8
39.5	2.3	2.1	2.8	1.3	147	145	145	144	145
0.97	0.77	0.66	1.00	0.59	1.7	1.1	1.9	2.5	2.4
40.5	1.1	1.5	0.4	0.7	152	147	148	148	145
0.39	1.29	0.49	0.96	1.33	3.1	1.3	0.8	0.5	0.9
h. s.	s.	s.	h. s.	h. s.	—	—	h. s.	h. s.	h. s.
—	—	s.	s.	—	—	s.	s.	s.	—
h. s.	—	—	—	—	h. s.	h. s.	h. s.	h. s.	h. s.
h. s.	—	s.	h. s.	h. s.	—	—	—	s.	—
h. s.	—	—	—	—	—	—	—	—	—

observations; two patients were receiving 5 mg. DCA in oil daily during the study.* At the end of the first hour following hydration, there was a percentage increase in EV (+3.1 per cent) which probably was significantly greater than normal ($p<0.05$), but the increase in plasma hydration (+1.5 per cent) was slightly but not significantly less than normal. The percentage increase in BV was of normal magnitude, but a greater proportion of the intravascular water had entered the cells. An excessive fall in serum base concentration (4 meq./L.) was observed. Although calculated percentage changes in BV during the remainder of the test were not significantly different from normal, the mean values tended to be somewhat in excess of the normal pattern. This excess intravascular water was accounted for by a statistically significant percentage increase in EV ($p = <0.05, <0.01, <0.01$). PV showed a progressive percentage increase equal to that observed in normal subjects. The serum base concentration remained depressed throughout the study and paralleled the persistent overhydration of the red cells. The maintenance of an abnormally high urinary specific gravity throughout the test suggested failure to achieve normal dilution of the urine, and a significant depression in urine flow was observed.

*The influence of DCA therapy on the results of the water test in patients not receiving cortisone has been evaluated. The results in 7 of the patients who had not received DCA were compared statistically to 5 patients receiving DCA in therapeutic doses, 3 of whom were included in the group of 10 subjects not receiving cortisone therapy. Additional DCA studies were performed on 2 of the subjects in Group 2A who also had water tests before DCA. There were no statistically significant differences in the erythrocyte responses between the groups, and no improvement in water diuresis was observed. Therefore the effects of DCA either in patients not on cortisone or as a factor influencing the results observed in cortisone-treated subjects probably could be disregarded.

2. PATIENTS RECEIVING 12.5 TO 25 MG. DAILY CORTISONE THERAPY. (Table I, *2B*, and Fig. 1):

Two men and four women were included in this group. They were receiving cortisone regularly prior to the test.* Cortisone was withheld on the day of study. There was a normal percentage increase in EV and a slight percentage decrease in PV at the end of the first hour resulting in only a slight increase in BV (+0.5 per cent). However, these values were not significantly different from normal. PV expansion thereafter tended to be slightly but not significantly greater than normal. The normal percentage increase in EV observed in the first hour persisted through the second and third hours,

TABLE I, B

GROUP	DIAGNOSIS	NO.	CUMULATIVE WATER EXCRETION % 1	2	3	4	OVERNIGHT*	URINARY SPECIFIC GRAVITY FASTING†	1	2	3	4
1.	Normal subjects	13	11	50	69	74	1.024	1.022	1.009	1.002	1.005	1.012
	S.E.M.		2.2	3.9	4.2	4.7	.0019	.0017	.0014	.0006	.0014	.001
2.	Addison's disease											
	A. No cortisone	10	5	13	19	26	1.013	1.015	1.016	1.010	1.008	1.009
	S.E.M.		1.4	1.9	2.8	4.2	.0028	.0023	.0018	.0023	.0019	.002
	B. 12.5 to 25 mg. cortisone	6	4	23	42	59	1.014	1.016	1.013	1.002	1.002	1.004
	S.E.M.		1.0	2.9	4.9	8.5	.0024	.0021	.0031	.0008	.0014	.002
	C. 100 to 200 mg. cortisone	9	10	45	68	75	1.015	1.018	1.011	1.001	1.002	1.007
	S.E.M.		3.4	5.1	7.5	7.7	.0021	.0027	.0029	.0004	.0006	.001
3.	Adrenalectomy											
	A. Before operation	5	6	14	31	40	1.015	1.016	1.014	1.006	1.004	1.003
	S.E.M.		0.9	2.1	9.6	10.5	.0036	.0027	.0040	.0022	.0018	.001
	B. After operation	5	7	32	48	58	1.010	1.013	1.011	1.003	1.006	1.006
	S.E.M.		2.0	7.9	12.2	13.2	.0040	.0009	.0004	.0009	.0027	.001
Level of Significance of Difference Between Means ("t" Test)												
Groups	1 and 2A		s.	h. s.	h. s.	h. s.	h. s.	s.	h. s.	h. s.	—	-
	1 and 2B		—	h. s.	h. s.	—	h. s.	s.	h. s.	—	—	h. s.
	1 and 2C		—	—	—	—	—	—	—	—	—	—
	1 and 3A		—	h. s.	h. s.	h. s.	s.	—	—	s.	—	h. s.
	1 and 3B		—	s.	s.	—	h. s.	h. s.	—	—	—	s.

*Overnight urine 10 P.M. to 7 A.M.
†Fasting urine 7 A.M. to 8:30 A.M.

at which time a difference from the normal values for these periods of borderline significance was evident ($p<0.05$). Dilution of the urine was delayed until the end of the second hour and, although urine flow rose somewhat, there was a significant depression of water excretion in the second and third hours ($p<0.01$). Serum base remained depressed through the third hour of the test denoting persistent plasma dilution. The moderate water diuresis persisted through the fourth hour so that at the end of the four-hour period of study, cumulative water excretion was of normal magnitude, serum base levels had returned toward normal, and EV declined to within normal range.

*Two subjects received 45 and 60 mg. DCTMA intramuscularly one month prior to study. One subject received 5 mg. DCA intramuscularly the day before, and 2 subjects received daily doses of 2.5 and 5 mg., respectively, for some time prior to study.

3. PATIENTS GIVEN 100 TO 200 MG. CORTISONE (Table I, *2C*, Fig. 1):

Nine patients were given large doses of cortisone. All of the subjects received 100 mg. by mouth 2 hours prior to study, and 4 received an additional 100 mg. ten and one-half hours before water administration.* The percentage increases in EV and PV one hour after hydration were within normal limits. Percentage PV changes thereafter were less than normal but did not differ significantly because of a wide variability in individual observations. This relative decline in plasma hydration was responsible for a similar decrease in BV, since EV returned to control values after the first hour. The normal response of the erythrocytes to hydration of the blood was associated with the restoration of a normal water diuresis. Although the initial serum base concentration was slightly below normal as in the untreated group, there was a normal decline with hydration and return toward control levels with diuresis.

C. *Bilaterally adrenalectomized patients* (Table I, *3A*, and *3B*, and Fig 1): Tests of water excretion were performed before and after total bilateral adrenalectomy on 4 men over 50 years of age with advanced prostatic carcinoma,

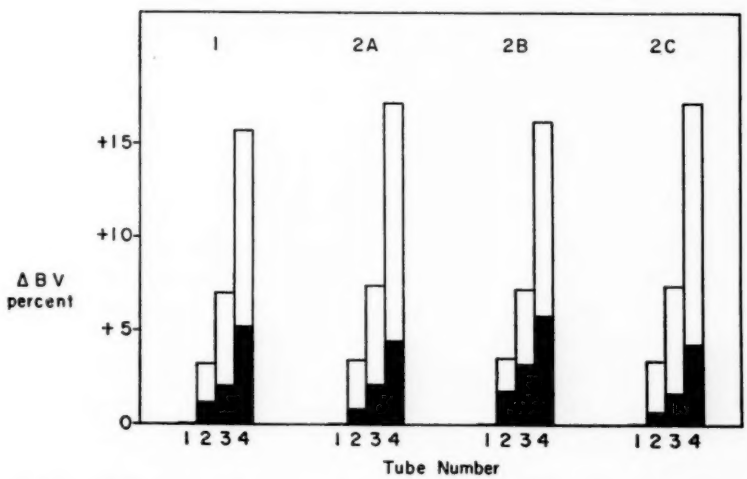

Fig. 2.—Distribution of intravascular water between plasma and erythrocytes following in vitro dilution of blood from normal and Addisonian subjects. Illustration of the proportions of the percentage changes in blood volume contributed to by increases in erythrocyte volume (black columns) and plasma volume (white columns).

$$\Delta \text{ BV per cent} = 100 \left(\frac{PV_2}{PV_1} (1\text{–Hct.}_1) + \frac{EV_2}{EV_1} (\text{Hct.}_1) - 1 \right)$$

See text for method of study.
Numerals above each section refer to corresponding groups in Table II.

and one 29-year-old man with severe hypertension. All of the patients with carcinoma had undergone bilateral orchiectomy prior to adrenalectomy. The results of the tests performed prior to adrenalectomy in these patients differed significantly in many respects from the response of normal subjects, resembling

*Four of these patients had received 30 to 60 mg. DCTMA intramuscularly within one week prior to study. Two subjects were receiving intramuscular DCA in oil at 2.5 and 5 mg. daily, respectively, at the time of study.

those seen in patients with Addison's disease. There was a statistically significant percentage increase in EV during the last 3 hours of the study. Percentage changes in PV after the first hour were less than normal but did not differ significantly from normal because of wide variations in individual values. The decline in urinary specific gravity was delayed, and dilution of the urine persisted through the fourth hour. The water diuresis was significantly depressed ($p<0.01$). Following adrenalectomy, all of the patients received 25 mg. cortisone by mouth daily. Three of the subjects were not receiving supplementary DCA therapy; the remaining two received 2 and 2.5 mg. intramuscularly daily. Both cortisone and DCA were withheld on the morning of study. The postoperative results were also compared to those of normal subjects (Table I, B, *3B*). Bilateral adrenalectomy and maintenance cortisone therapy caused slight improvement in water excretion. The erythrocyte response was reverted toward normal. A greater amount of the excess intravascular water was retained in the plasma in the second and third hours in comparison to the excess hydration of the cells observed preoperatively. There was no statistically significant difference, however, between the preoperative and postoperative results, which may have been influenced by the small size of the series.

II. *In Vitro Studies.*—In vitro dilution of whole blood as previously described was performed in 6 normal subjects and 10 patients with Addison's disease. Five of these patients were not receiving cortisone; the remainder were receiving 12.5 to 25 mg. cortisone daily as maintenance therapy. One-half milligram crystalline hydrocortisone was added to a separate series of tubes in 3 of the studies on untreated patients. The percentage changes in BV, PV,

TABLE II. PERCENTAGE CHANGES IN BV, PV, AND EV PRODUCED BY ADDITION OF WATER IN VITRO TO BLOOD OF NORMAL SUBJECTS AND PATIENTS WITH ADDISON'S DISEASE

			ΔBV %				ΔPV %				ΔEV %			
GROUP	DIAGNOSIS	NO.	HGB.$_1$*	2	3	4	1-HCT.$_1$*	2	3	4	HCT.$_1$*	2	3	4
1.	Normal subjects	6	16.0	3.2	7.0	15.7	52.9	3.8	9.3	19.7	47.1	2.6	4.5	11.2
	S.E.M.		0.39	0.24	0.14	0.20		0.91	0.38	0.42	1.05	0.28	0.44	0.63
2.	Addison's disease													
	A. No cortisone therapy	5	13.0	3.4	7.6	17.1	59.9	4.4	9.1	21.0	40.1	2.0	5.2	11.2
	S.E.M.		0.56	0.11	0.25	0.51		0.46	0.73	0.81	2.04	0.74	0.82	1.21
	B. 12.5 to 25 mg. cortisone daily	5	14.4	3.5	7.3	16.3	56.1	3.1	7.3	18.5	43.9	4.0	7.3	13.6
	S.E.M.		0.83	0.17	0.27	0.17		0.24	0.93	0.98	2.45	1.11	1.20	0.76
	C. No cortisone therapy, 0.5 mg. comp. F added to each tube†	3	12.6	3.6	7.4	17.4	61.8	4.6	9.3	21.1	38.2	1.9	4.3	11.4
Level of Significance of Difference Between Means ("t" Test)‡														
Groups 1 and 2A			—	—	—	—	—	—	—	—	—	—	—	—
1 and 2B			—	—	—	—	—	—	—	—	—	—	—	—

For method of calculation see text. All values for ΔBV, ΔPV, and ΔEV carry a + sign.
*Control (undiluted) specimen.
†Hydrocortisone (free alcohol).
‡See legend Table I.

and EV observed in these studies are shown in Table II (also see Fig. 2). Definite conclusions from these observations cannot be made because of the small number in each series; however, the results suggested that the erythrocytes of the patients with Addison's disease, when exposed to a hypotonic environment in vitro, did not exhibit an abnormal permeability to water with gradations in hemodilution which exceeded that induced in vivo. Furthermore, in the subjects studied the addition of a relatively large dose of hydrocortisone to the blood of patients with untreated Addition's disease did not alter the response of the cells.

DISCUSSION

The administration of a large volume of water to patients with Addison's disease not receiving cortisone is associated with the following abnormalities: (1) a marked and prolonged increase in erythrocyte volume despite a normal degree of plasma volume expansion; (2) an excessive and persistent depression of serum base concentration; (3) a failure to achieve normal dilution of the urine and a depressed water diuresis.

It has been suggested[8] that derangements 2 and 3 may be due to an antidiuretic mechanism not dependent on serum tonicity. Although antidiuretic assays have been reported to be positive during tests of water excretion in Addison's disease,[8, 9] these results must be viewed with some reservation at this time, since there is considerable doubt as to the reliability of the assay techniques employed.[10, 11] Evidence has been accumulated which demonstrates that the duration of action of physiologic amounts of Pitressin administered intravenously is not prolonged in adrenal insufficiency in man and not shortened by cortisone therapy.[12] These results would tend to argue against an increased sensitivity to or delayed inactivation of circulating ADH as the underlying disturbance. The possible role of a persistence of ADH secretion, despite overhydration and plasma hypotonicity, however, has not been eliminated, and there is some evidence that the diuretic effect of cortisone may, in part, be due to an inhibition of ADH secretion from the neurohypophysis.[13]

Since the calculated percentage changes in EV herein reported probably reflect alterations in erythrocyte hydration, an abnormal increase in at least a portion of the total intracellular volume accompanies the retention of administered water observed in patients with untreated adrenal insufficiency. Although the only body cells investigated in this study were erythrocytes, there is evidence to suggest that excessive hydration of other body cells may also be observed in adrenal insufficiency. Thus, a greater than normal uptake of water by the body cells of adrenalectomized rats given large water loads has been reported by Frost and Talmage.[14] An increase in water content of red cells alone[15] and of both red cells and muscle cells[16] has been demonstrated in experimental adrenal insufficiency. Also, an increase in mean corpuscular volume and in erythrocyte volume (calculated from the published Hct. and Hgb. results), accompanied by a decrease in thiocyanate space, has been observed in untreated adrenalectomized dogs.[17] These changes were reversed with adrenal extract therapy. In view of these findings, it is probable that the

excessive percentage increases in EV observed following water administration in the present study reflect an abnormality in fluid distribution which is a characteristic of adrenal cortical insufficiency. In this regard, a significantly greater than normal percentage increase in EV was observed one hour after hydration in the untreated patients, at a time, preceding diuresis, when the volume of retained water and the percentage increase in PV were equal to those of normal subjects. These percentage changes in EV, therefore, probably reflect an abnormal permeability of the erythrocytes to water. Since water is freely diffusible between the vascular and extracellular compartments, it is theoretically possible that there is an excessive uptake of water by the other body cells as well. The faulty distribution of water may predispose to a poor diuresis, however, rather than be merely secondary to a failure of proper renal excretion of water. The fact that the percentage increase in PV observed in these subjects was within normal range suggests that most of the retained water which would be excreted normally through the kidneys had been transferred to extravascular, possibly intracellular compartments, where it may not be available for proper excretion. Cortisone therapy may prevent overhydration of cells and tend to keep the fluid in the extracellular compartment, where it should be more readily excreted by the kidneys.

The observation that the erythrocytes of patients with Addison's disease evidenced a normal increase in volume when a hypotonic expansion of plasma volume was produced in the test tube suggests that the abnormal erythrocyte changes observed during tests of water excretion may not be due to an intrinsic abnormality of the red cells themselves. It is more likely that this phenomenon is mediated by unknown factors which promote the entrance of water into erythrocytes contained only within the vascular system. This hypothesis need not imply a primary defect in the erythrocytes. Since there is little doubt that red cells maintain osmotic equilibrium with the plasma, it is possible that the excessive hypotonicity of the plasma is primarily responsible for the abnormal degree of erythrocyte hydration observed in these patients. Since there was a close relationship between improvement in the erythrocyte response, restoration of a normal diuresis, and a return of serum base concentrations to control levels, an attractive yet unsubstantiated hypothesis may be that disorders in the secretion or metabolism of the antidiuretic hormone may be primarily responsible.

The failure of hydrocortisone to alter the erythrocyte response in vitro suggests that the adrenal steroids may not directly influence the entrance of water into the red cells. However, this hypothesis does not necessarily rule out a direct in vivo action which may require transfer to extravascular sites or renal excretion of red cell water or electrolytes for promulgation of steroid effects. On the other hand, these hormones may alter the distribution of intravascular water in vivo indirectly, either by influencing the secretory activity of the neurohypophysis or the peripheral metabolism of ADH. In the light of our present knowledge, the former mechanism would be more likely.

Although the preoperative results in patients subjected to total bilateral adrenalectomy resembled those of patients with untreated Addison's disease,

it is not possible to ascertain whether these abnormalities reflect a disturbance in endogenous adrenal cortical secretion or were due to factors other than adrenal steroids operating in these chronically ill patients. In any event, it is obvious that the defect in the distribution of intravascular water observed in patients with untreated Addison's disease is not pathognomonic of this disorder, and may be observed in other chronic illnesses not associated with apparent hypocorticism. This too would imply that the abnormal distribution of water is related to an extra-adrenal factor which may be the common denominator.

SUMMARY

1. Tests of water excretion were performed in patients with adrenal cortical insufficiency before and after cortisone administration. Percentage changes in blood, plasma, and erythrocyte volumes were calculated from hourly changes in hematocrit and hemoglobin measurements for a four-hour period following the oral administration of 20 ml. water per kilogram of body weight.

2. There was an excessive and prolonged percentage increase in erythrocyte volume following hydration in patients with Addison's disease not on cortisone. Percentage changes in plasma volume, however, were within normal range. The abnormal erythrocyte response was accompanied by a marked fall in serum base concentration, a subnormal decline in urinary specific gravity, and a depressed water excretion. Patients receiving 25 mg. maintenance cortisone therapy had slightly abnormal percentage changes in erythrocyte volume following hydration which paralleled an abnormal delay in water excretion. Patients given 100 to 200 mg. of cortisone prior to hydration showed completely normal responses.

3. Preoperatively, 5 men who underwent bilateral total adrenalectomy demonstrated findings similar to those of patients with Addison's disease not receiving cortisone. Postoperatively, some improvements in the erythrocyte response and the water diuresis were observed while the patients were on 25 mg. of maintenance cortisone daily.

4. Addisonian red cells did not swell excessively when a hypotonic increase in plasma volume was induced in vitro. Hydrocortisone added in vitro did not alter the permeability of the erythrocytes to water. The suggestion is made that the abnormal changes in erythrocyte volume observed in vivo may be due to an extraerythrocytic mechanism not dependent primarily on corticoid deficiency, but rather one which may be related to abnormalities in the secretion or metabolism of the antidiuretic hormone.

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CHARACTERIZATION OF STRAINS OF INFLUENZA VIRUS ISOLATED IN BOSTON IN 1943-1944

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A PREVIOUS paper[1] from this laboratory presented a detailed report concerning the isolation of viruses and serologic studies made during and after the height of the epidemic of influenza that occurred in Boston in December, 1943. Difficulties were encountered at that time in the classification of several of the strains, particularly those isolated from postepidemic cases, by inhibition of chicken cell agglutination and by neutralization tests in mice, with use of immune rabbit sera prepared against the PR8 strain of influenza A and the Lee strain of influenza B. Further attempts at classification by cross-neutralization tests in mice and also by cross-reactions in agglutination-inhibition tests with rabbit antisera yielded results that were not satisfactory and were soon abandoned; they did, however, show differences among the strains, some being similar to PR8.[2]

There are now many studies utilizing different methods and indicating considerable antigenic differences among strains of influenza A and B viruses isolated at different times and in many countries.[3-12] In this laboratory, the use of immune rabbit sera in cross-neutralization tests in chick embryos has permitted a satisfactory delineation of antigenic differences among related strains isolated at the same or different times.[13-15] The present report concerns the use of this method in the characterization of the strains isolated here in 1943-1944.

MATERIALS AND METHODS

Viruses.—The following strains were included in the complete in ovo cross-neutralization tests: (a) 9 of the strains isolated in 1943-1944; 2 of these were obtained from lungs at autopsy, the others from throat washings. The strain designations formerly used[1] are retained; Strains A1 and 4 were isolated in 1943 on December 13 and 14, respectively, the other 7 were isolated from materials obtained between February 1 and March 12, 1944. (b) Three "standard" strains of influenza A, namely, PR8, isolated in 1934, Weiss isolated from a sporadic case in Michigan on May 28, 1943,[16] and FM1 (A prime) isolated in 1947.[17] (c) Two "standard" strains of influenza B, namely, Lee and Bon, the latter isolated in Australia in 1943.[18] (d) Three A strains isolated in this laboratory, EB and DW in 1947 and Coop in 1948.[15] The PR8, Weiss, and Lee strains were originally isolated by passage in ferrets and mice but have received many allantoic passages in chick embryos prior to use in the present studies. The remaining strains were

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all originally isolated in eggs and have also received many allantoic passages; the strains isolated in this laboratory in 1943-1944 were used after about 25 egg passages, those isolated here in 1947 and 1948 were used after 16 to 18 such passages.

Methods.—The method used for in ovo neutralization was essentially that of Hirst,[19] with fourfold dilutions of serum (the lowest dilution being 1:4) and a constant amount of virus injected allantoically. Rabbit antisera were prepared as in previous studies.[14] All of the antisera were titrated simultaneously with each virus, and the infectivity titer (ID_{50}) of the inoculum of that virus was determined at the same time. Normal rabbit serum, obtained before immunization, was included along with the homologous immune serum each time a virus was used in these tests. The titers are expressed as the reciprocals of the initial dilution of serum at the 50 per cent end point of inhibition of infection as estimated by the method of Reed and Muench.[20]

Antigenic Comparisons.—Antigenic ratios were calculated from the titers by the formula recommended by Chu, Andrewes, and Gledhill,[7] and by Archetti and Horsfall,[21] viz., $R = \sqrt{r_1 \times r_2}$, where r_1 and r_2 represent the heterologous titer divided by the homologous titer for viruses r_1 and r_2, respectively. For convenience, these ratios are expressed in per cent, i.e., as $R \times 100$.

RESULTS

The normal (preimmunization) rabbit sera all failed to neutralize any of the strains in the lowest titer used, namely, 1:4. In addition, none of the strains isolated here in 1943-1944 were neutralized by antisera for any of the influenza B strains or by those against any of the influenza A strains isolated here in 1947 and 1948, and conversely, none of the antisera against the 1943-1944 strains under study neutralized any of the B viruses or any of the A strains isolated here in 1947 and 1948. These results are, therefore, not presented. The titers obtained in the complete cross-neutralization tests done with the 1943-1944 strains and with PR8, Weiss, and FM_1 are shown in Table I. The antigenic ratios calculated from these titers are also shown in the lower half of this table.

From the neutralization titers and their ratios the strains isolated in 1943-1944 appeared to fall into 3 antigenic groups. In one group are Strains 67, 70, and 71 which were antigenically very similar to each other and to PR8; these strains showed complete cross- neutralization among themselves and with PR8 but they showed only "asymmetric" cross-neutralization with Weiss in that the viruses were all neutralized by Weiss antiserum but their antisera did not neutralize the Weiss virus. A second group comprised Strains A1, 4, 66, and 68, which were antigenically very similar to each other and to Weiss but clearly distinct from PR8 and from the strains of the first group; these strains showed marked cross-neutralization among each other and with Weiss, but they were neutralized to a less extent or not at all by the PR8 antiserum and their antisera, like the Weiss antiserum, failed to neutralize PR8 virus. The 2 remaining strains, A2 and 69, were similar to each other and each reacted about equally with the strains of the other 2 groups and with both PR8 and Weiss so that, judging from their antigenic ratios, they may be considered as intermediate and as sharing the antigenic properties of both of the first 2 groups. Unfortunately, the low homologous

neutralizing titers of these 2 antisera and of some of the others which had been stored for several years tended to blunt these comparisons to some extent. All of the strains were clearly distinct from FM_1.

The 2 strains that were classified as intermediate were selected for special study to determine, if possible, whether they represented single strains sharing antigens with PR8 and Weiss or were each mixtures of 2 viruses, one resembling PR8 and the other similar to Weiss. Two methods of approach were used, namely, (1) isolation and identification of virus from end-dilutions of the strains to determine whether the minimum infecting amounts of virus were antigenically homogeneous or might reveal both varieties from different eggs, and (2) identification of virus surviving neutralization separately with antisera against each of the antigenic varieties of virus.

TABLE I. IN OVO CROSS-NEUTRALIZATION TITERS WITH STRAINS OF INFLUENZA A AND THEIR CORRESPONDING ANTISERA

VIRUS	YEAR	INOCULUM ID_{50}	IMMUNE RABBIT SERA*											
			FM_1	WEISS	PR8	67	70	71	A2	69	A1	4	66	68
FM_1	1947	667	**645**	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4
Weiss	1943	1670	<4	**1630**	<4	<4	<4	6	<4	40	16	128	16	128
PR8	1934	158	<4	128	**256**	32	4	128	<4	102	<4	8	<4	<4
67	1944	1630	<4	512	6500	**512**	512	1024	40	1024	4	128	4	32
70	1944	667	<4	128	512	64	**32**	128	10	186	<4	26	<4	16
71	1944	250	<4	162	1024	102	64	**646**	32	256	<4	32	<4	32
A2	1944	333	<4	256	128	32	16	64	**32**	256	<4	64	<4	32
69	1944	1670	<4	186	8	<4	<4	8	4	**32**	<4	26	<4	32
A1	1943	500	6	1024	32	<4	4	8	8	64	**51**	256	32	256
4	1943	333	8	2580	32	<4	<4	6	6	64	26	**408**	89	162
66	1944	167	4	2048	16	<4	<4	6	4	32	40	128	**32**	162
68	1944	333	<4	128	<4	<4	<4	<4	<4	16	8	32	8	**64**

Titer-Ratios Calculated From Values Given Above and Expressed as "R × 100"

FM_1	100											
Weiss	<0.4	100										
PR8	<1	3	100									
67	<0.7	<5	124	100								
70	<3	<10	50	141	100							
71	<0.6	3	89	57	63	100						
A2	<3	<14	<25	28	39	31	100					
69	<3	37	32	<50	<85	31	100	100				
A1	<3	44	<10	<3	<10	<3	<14	<40	100			
4	<1	71	5	<5	<9	3	17	36	57	100		
66	<3	79	<9	<3	<13	<3	<13	<35	88	94	100	
68	<2	40	<3	<6	<18	<6	<25	50	79	45	80	100

*All titers are expressed as the reciprocal of the initial dilution of serum. Normal (pre-immunization) serum from each rabbit was tested simultaneously with homologous immune serum; the titer was <4 in every instance. Homologous titers are in bold type.

In one experiment, therefore, serial dilutions of Strains A2 and 69 in the region of the end-points of their egg infectivity were inoculated allantoically into eleven-day embryos. After 48 hours, 3 of the 10 eggs inoculated with the 10^{-9} dilution of Strain A2, and 2 of 10 inoculated with the 10^{-7} dilution of Strain 69 were found to be heavily infected. The in ovo neutralizing titers of PR8 and Weiss antisera were then determined for the viruses contained in

allantoic fluid of each of these eggs. The results are given in the upper part of Table II. They show that all 5 of the viruses grown from the end-dilutions of A2 and 69 were neutralized in about equal titers by the PR8 and the Weiss antisera, the actual titers varying with the number of ID_{50} used for the inoculum. In this respect, then, the viruses from all 5 of these eggs resembled the PR8 strain. These end-dilutions, therefore, did not yield virus reacting antigenically like Weiss.

TABLE II. IN OVO NEUTRALIZATION BY ANTI-PR8 AND ANTI-WEISS RABBIT SERA

STRAIN	SOURCE OF VIRUS FOR INFECTION	NO. OF ID_{50}	NEUTRALIZING TITER		ANTIGENIC RELATION
			ANTI-PR8	ANTI-WEISS	
A2	Each of 3 eggs infected among 10 inoculated with 10^{-9} dilution	No. 1 $>10^4$	20	14	PR8
		No. 2 500	128	128	PR8
		No. 3 160	100	100	PR8
69	Each of 2 eggs infected among 10 inoculated with 10^{-7} dilution	No. 1 $>10^5$	10	14	PR8
		No. 2 2340	64	32	PR8
A2	Egg infected with 2040 ID_{50} of A2 + 1:16 dilution of anti-PR8 rabbit serum	234	<4	51	Weiss
A2	Egg infected with 2040 ID_{50} of A2 + 1:32 dilution of anti-Weiss rabbit serum	158	33	32	PR8
69	Untreated virus	333	<4	32	Weiss
69	Egg infected with 6700 ID_{50} of 69 + 1:32 dilution of anti-Weiss rabbit serum	343	32	22	PR8

In the second experiment an attempt was made to determine whether more than one antigenic variety of virus could be isolated from Strains A2 and 69 by neutralization with specific PR8 and Weiss antisera. Strains A2 and 69, alone and mixed with excesses of PR8 and Weiss antisera, were inoculated into groups of eggs, and the allantoic fluids of some of the individual eggs that were found to be heavily infected with virus after 48 hours were used in neutralization tests with PR8 and Weiss antisera. The results of 4 such tests are shown in the lower part of Table II. They show that Strains A2 and 69 each contained at least 2 antigenically distinguishable varieties of virus; one was neutralized to about the same extent by both PR8 and Weiss rabbit antisera and the other was neutralized by the anti-Weiss but not by the anti-PR8 serum. The former, as seen from Table I, is the characteristic of the PR8 strain and also of Strains 67, 70, and 71, whereas the latter is antigenically similar to the Weiss strain and also to Strains A1, 4, 66, and 68.

The identification of 2 distinguishable antigenic varieties of virus from each of the Strains A2 and 69, therefore, strongly suggested laboratory contamination—at least in these 2 strains. Both of them, like all of the others, had been subjected to a large number of passages in eggs and such contamination was of course possible, particularly with the PR8 virus, which was being used in the laboratory continuously during the period when these strains were originally isolated and passaged.

It is of interest that of the strains used in the cross-neutralization test shown in Table I, only 2 were obtained in December, 1943, during the height of the epidemic and both of them were antigenically similar to Weiss. Of the 7 postepidemic strains isolated in 1944, only 2 (Nos. 66 and 68) resembled Weiss, whereas 3 others (Nos. 67, 70, and 71) were antigenically similar to PR8, and the remaining 2, which were studied in greater detail, were shown to be mixtures of these 2 antigenic varieties. It thus seemed likely that those strains that resembled PR8 and which were found either alone or mixed with those of the Weiss variety were indeed laboratory contaminants.

In the hope of obtaining more possible confirmation, attempts were made to reisolate viruses from all of the original throat washings and lung suspensions that were collected during the 1943-1944 epidemic. These had all been stored at −70° C. (in a dry-ice box) in rubber-stoppered Lusteroid tubes sealed over with Scotch tape. No preservatives or antibiotics had been added before storage. The results of these attempts are shown in Table III. Only 3 of the 7 washings that had been stored in this manner yielded viruses in these attempts; no virus could be isolated on 1 or more attempts from the remaining washings or from the 2 lung suspensions that were available and materials from Patients 70 and 69 were no longer available. Original Virus 4 was isolated from unfiltered but not from filtered washings[1]; only the latter was available for the recent attempts.

TABLE III. RESULTS OF ATTEMPTS TO REISOLATE INFLUENZA VIRUSES FROM THROAT WASHINGS AND LUNGS OF PATIENTS OBSERVED DURING THE INFLUENZA EPIDEMIC OF 1943-44

PATIENT NO.	DATE OF WASHING	CHICK-EMBRYO INOCULATIONS			PASSAGE USED TO IMMUNIZE RABBITS	HI TITERS OF RABBIT ANTISERA NORMAL/IMMUNE
		DATE OF INITIAL INOCULATION*	PASSAGE	RESULT		
1	12/ 3/43	4/22/55	Al_2	0		
		5/13/55	Am_1	+	Am_1Al_3	32/2048
2	12/ 4/43	7/ 8/55	Am_2	0		
3	12/ 7/43	4/22/55	Al_2	0		
		7/ 8/55	Am_2	0		
4	12/14/43	4/22/55	Al_2	0		
		5/ 6/55	Am_3	0		
66	2/ 8/44	4/22/55	Al_2	0		
		5/13/55	Am_2	0		
67	2/11/44	4/22/55	Al_2	+	Al_3	64/8192
68	2/29/44	4/22/55	Al_1	+	Al_2	16/4096
71	3/12/44	5/13/55	Am_2	0		
		7/ 8/55	Am_2	0		
A1	12/13/43	6/10/55	Am_3	0		
		6/30/55	Am_2	0		
A2	2/ 1/44	5/20/55	Am_1	Grossly contaminated by fungus		
		6/10/55	Am_2			
		6/22/55	Am_4			

*During present attempts; results of earlier ones previously reported.[1]

Abbreviations: A1 and A2 are patients who died and whose lungs obtained at autopsy were used; A1 = allantoic and Am = amniotic inoculations, the subscripts denoting the number of passages by that route before hemagglutinin was demonstrated in the allantoic fluid (result +) or before attempts were abandoned because such hemagglutinin was not demonstrated (0). HI = hemagglutination-inhibition by chicken erythrocytes. The "normal" serum denotes preinoculation serum obtained from the same rabbit.

The reisolated strains were from Patients 1, 67, and 68; the first of these was isolated from a washing obtained in December, 1943, during the height of the epidemic and the other 2 were from the postepidemic period. The strain originally isolated from Patient 67 was shown in Table I antigenically similar to PR8 and the original isolation from No. 68 resembled Weiss. The antihemagglutinin titers of the acute and convalescent sera of the 3 patients from whose throat washings these viruses were reisolated are listed in Table V; these tests, both the original ones and the repeats, had been done with earlier passages of both homologous and the PR8 viruses, but the sera were heated for 20 minutes at 60° C. when the tests were repeated.

TABLE IV. RESULTS OF IN OVO CROSS-NEUTRALIZATION TESTS WITH FRESHLY REISOLATED STRAINS OF THE 1943-1944 EPIDEMIC, CERTAIN OTHER TYPE A STRAINS, AND THE CORRESPONDING ANTISERA

VIRUS	YEAR	INOCULUM ID_{50}	IMMUNE RABBIT SERA*							
			PR8	WEISS	1	67	68	FM_1	13	9
PR8	1934	158	**512**	46	<4	7	6	<4	<4	<4
Weiss	1943	1580	6	**162**	64	124	89	<4	6	<4
1	1943	500	<4	32	**162**	100	162	<4	<4	<4
67	1944	158	10	162	1024	**725**	1024	11	64	11
68	1944	231	<4	64	512	161	**204**	<4	<4	<4
FM_1	1947	214	<4	<4	<4	<4	4	**256**	41	10
13	1953	107	<4	<4	<4	<4	<4	<4	**410**	64
9	1955	346	<4	<4	<4	<4	8	8	38	**410**
Titer-Ratios Calculated From Values Given Above and Expressed as "R × 100"										
PR8			100							
Weiss			2	100						
1			<1	59	100					
67			1	41	93	100				
68			<2	41	63	105	100			
FM_1			<1	<2	<2	<2	<2	100		
13			<1	<5	<2	<3	<1	<4	100	
9			<1	<2	<2	<1	<2	3	12	100

*See footnote to Table I.

The third allantoic passage of each of the 2 newly isolated strains was used to prepare antigens and rabbit antisera which were then employed in cross-neutralization tests in ovo. The PR8, Weiss, and FM_1 strains were again included, and, in addition, Strains 13 and 9, isolated in 1953 and 1955, respectively, and each antigenically characteristic of other strains isolated in this laboratory during the same season* were added. The results are given in Table IV. The 3 newly isolated strains were antigenically very similar to each other and to Weiss and were clearly distinct from PR8 with which they exhibited an "asymmetric" cross-neutralization similar to that shown by the Weiss strain. All of these strains, in turn, were antigenically entirely distinct from FM_1 and from the 1953 and 1955 strains. The last 2 strains were also distinct from FM_1 and from 1943-1944 strains but showed some antigenic relationship to each other.

*Detailed antigenic comparisons of Strains 13 and 9 with other Type A strains are included in other recent reports.[43, 44]

The sera of the patients all showed some rises in titer of antihemagglutinins to PR8, or to the homologous virus or to both, but failed to show any change in titer with the Lee strain not shown in the table. Patient 1 had a marked rise in titer to PR8 in 2 tests and a rise of border-line significance with the homologous strain, whereas the sera from Patient 68 showed no significant rise in the original tests with both PR8 and the homologous strains but a fourfold rise in titer was elicited with both strains in subsequent tests with sera heated for 20 minutes at 60° C., which may have reduced the titer of nonspecific inhibitor.

TABLE V. RESULTS OF HEMAGGLUTINATION-INHIBITION TESTS ON SERA OF PATIENTS FROM WHOM VIRUSES WERE REISOLATED

PATIENT NO.	DATE OF SERUM	TITER OF ANTIHEMAGGLUTININS*			
		PR8		HOMOLOGOUS	
		ORININAL[1]	REPEAT	ORIGINAL[1]	REPEAT
1	12/ 3/43	16	16	8	–
	12/ 7	8	8	8	–
	12/24	512	256	64	–
	12/31	512	128	128	–
	2/28/44	512	64	256	–
67	2/11/44	16	4	<4	–
	2/17	16	4	<4	–
	3/ 2	16	4	<4	–
	3/22	16	4	8	–
68	2/29/44	64	64	64	16
	3/ 7	128	128	64	32
	3/14	128	256	64	64
	3/31	128	256	64	64
	4/12	128	128	64	–

*Recriprocal of serum dilution.
– = not done.

DISCUSSION

Strains of influenza virus of the same type isolated during a single epidemic, or even during the same season in widely separated places, have generally shown identical or very close antigenic relationships.[3, 7, 9, 10, 12, 22-25] However, there is also evidence that antigenically distinct strains, or strains which are related but still distinguishable from one another, may be demonstrated during the same epidemic, either at the same time or within a brief interval.[5, 13, 26] In several instances both A and B strains have been isolated from different patients in the same outbreak* and have been associated with serologic evidence of infection with these respective viruses.[7, 12, 26-31] Successive infections with A and B strains in the same patient have also been authenticated.[30, 31] Most of the evidence, however, points to a progressive change in the antigenic patterns of influenza viruses isolated in different years, particularly among A strains[8-10, 32] but also among B strains.[10-12, 33, 34]

In the present study, 2 antigenically distinct groups of strains were distinguished, one closely resembling PR8 and the other very similar to Weiss; in addition, 2 strains seemed to be related to both these groups. The 2 latter

*The most recent experience with concurrent outbreaks of influenza A and B infections was in the winter of 1955.[44]

strains and the 3 that resembled PR8 were all isolated in the postepidemic period, whereas of the 4 strains that resembled Weiss, 2 were isolated during the height of the epidemic and the others were obtained in the postepidemic period. Strains antigenically resembling PR8 have also been isolated by others during outbreaks along with dissimilar strains of influenza A in 1943[35] and even more recently.[36, 37]

The antibody response of most adult patients with clinical influenza has not proved useful in differentiating strains within the same type, even when wide differences in the antigenic composition of the strains can be demonstrated[15, 31, 34, 38-40]; such differentiation, however, has been possible at times in the convalescent serum of children.[31, 34, 38, 39] It is well known, however, that patients vary in their quantitative antibody response to infection with the same virus, and patients have occasionally been encountered in whom it has not been possible to demonstrate a significant rise in antibodies to the strain of influenza isolated from their own throat washings.[41] It is of interest in connection with the strains isolated here in 1943-1944 that all of the recovered patients from whom influenza viruses were isolated during the height of the epidemic developed significant and usually quite large increases in titer of antibodies both to PR8 and to their own strains, and the increases in titer to the former were of greater magnitude than to the latter in some of these patients. None of the postepidemic cases developed increases in titer of similar magnitude, either to their own strains or to PR8,[1] although fourfold increases in titer to the homologous strains were demonstrated in the sera of the 2 postepidemic cases from which viruses were reisolated and in some of the others.

The possibility that patients who fail to develop demonstrable antibodies against their own strain may represent transient and noninfected carriers has been mentioned in connection with the 1944 cases under study.[1] However, an alternative explanation in these and similar cases must be considered. Andrewes and his co-workers[35, 37, 42] have emphasized the importance of considering the influenza virus as a possible laboratory contaminant and of certain influenza viral infections as "laboratory pick-ups." This is particularly pertinent when dealing with strains that are antigenically similar to those which are used extensively in the particular laboratory concerned and different from other strains that are being isolated at that time. In connection with the strains being considered in the present study it should be noted that, whereas the PR8 strain had been in constant use in this laboratory during the time when the original isolations and egg passages were made, the Weiss strain did not become available in this laboratory until more than 2 years after all the 1943-1944 strains had completed their egg passages and been stored.

Andrewes and Glover[35] recovered 2 viruses in 1943 which proved to be serologically identical with their stock laboratory Strain PR8 and also were very highly virulent for mice; they suspected laboratory contamination, although one of the strains was isolated after no work had been done in the laboratory for 17 days. Isaacs and Andrewes[37] identified a few of the strains

sent to the World Influenza Center during 1950-1951 as similar to the classic PR8; they expressed the opinion that PR8 and WS (isolated in 1933) strains have entirely disappeared and that recent strains resembling them are likely to be laboratory pick-ups. Hilleman and co-workers,[36] on the other hand, interpreted their observations as indicating that the outbreak of influenza among Eskimos in Victoria Island in April, 1949, was caused by 2 different influenza viruses, one of which resembled PR8 and the other of which resembled FM_1, the former being considered an indication of the recurrence of the earlier prototype. Magill and Jotz[10] cited the circumstances in connection with 2 strains in their study in support of the contention of Isaacs and Andrewes that strains isolated from laboratory workers may be "pick-ups."

The mouse virulence of the strains isolated in 1943-1944 was not helpful in the differentiation of contaminants inasmuch as both of the laboratory strains, PR8 and Weiss, were highly virulent in spite of the fact that they had been subjected to numerous egg passages since their last contact with mice. Although none of the 1943-1944 strains used in this study had been passed in mice, they too were virulent, some of them only slightly less than PR8 or Weiss. The LD_{50} of the allantoic fluid viruses was determined for most of the strains used in this study: for PR8 and Weiss this was about 10^{-5}; for the original Strains 67, 71, and A2 it was about 10^{-4}; for the original Strains 4, 66, and 69 it was $10^{-1.5}$, and for the recently isolated Strains 1, 67, and 68 it was 10^{-4}, 10^{-2}, and 10^{-4}, respectively. The last 3 strains had had only 3 or 4 passages in eggs, the others had received 25 or more of such passages. Strains from the most recent epidemics were either totally avirulent in that they failed to produce significant lesions when allantoic fluid virus concentrates were injected intranasally, or they were infectious in dilutions no greater than 10^{-1} of these concentrations. Serial passages to enhance virulence were not attempted.

The first antigenic comparisons presented in Table I were subject to 2 alternative interpretations. Considered at their face value, they could be interpreted as indicating that 2 antigenically distinct strains of influenza virus were prevalent in Boston during and after the epidemic of influenza of December, 1943; one of these was similar to PR8 and the other similar to Weiss, and that intermediate strains sharing the antigenic properties of both were also present. Alternatively, and particularly in view of the failure to demonstrate significant antibody rises in some of the postepidemic cases, these data may be interpreted as indicating that the epidemic strains were antigenically similar to Weiss and that laboratory contamination with PR8 could account for those strains that were antigenically clearly similar to PR8 and for the 2 mixed strains A2 and 69. The recent reisolation of strains from 3 washings and the demonstration that all 3 of these new isolates clearly resembled Weiss, in spite of the fact that one of them (No. 67) was from a washing the original isolate of which was considered to be clearly like PR8, supports the latter alternative. Unfortunately, the lung suspension from Patient A2 and the throat washings from Patients 69, 70, and 71 either failed to yield viruses in the recent attempts or were no longer available so that similar evidence could not

be obtained with respect to these strains. Nevertheless it seems quite reasonable to conclude that all of those postepidemic strains that antigenically resembled PR8 represent laboratory contaminants and that Strains A2 and 69, as used in the antigenic comparisons, were probably mixtures of the epidemic strain with a similar contaminant. The time and the manner in which the contamination took place in these instances, as in those recorded by others, is not clear.

In this connection, it should be noted that only 2 of the strains included in this study were isolated from hospital personnel, both of them interns on the same hospital wards as some of the other patients from whom virus isolations were made; none were obtained from laboratory workers. The postepidemic strains were originally isolated, in general, in the same manner as the earlier ones, and this was done by several methods, both in eggs and in mice (although only the egg strains were used in these studies) and, in some instances, on more than one occasion from the original washings.[1] Moreover, the coincidence of strains resembling PR8 being isolated both in this laboratory and in England during the same year is interesting, although not necessarily significant in establishing their etiologic role. It is also of interest to note that viruses resembling PR8 were not encountered among strains subsequently isolated from other epidemics studied in this laboratory through the winter of 1949,[14, 15] but they were during the winter of 1950.[45]

These findings help to clarify the inconsistencies presented in the earlier report. They are presented to emphasize the importance of considering the possibility of laboratory contaminations whenever such inconsistencies are noted, and the need for carrying out studies that may help to identify them whenever feasible.

SUMMARY AND CONCLUSIONS

Studies were made of the antigenic relationships of strains of influenza virus isolated in embryonated hen's eggs during and after the influenza epidemic that occurred in Boston in December, 1943; these strains had previously been subjected to many egg passages. By the use of in ovo neutralization tests with immune rabbit sera, these strains fell into 3 antigenic groups: one resembled PR8, the 1934 strain of influenza A; the second was similar to Weiss, a strain of influenza A isolated in Michigan in May, 1943, and the third appeared to be antigenically related to both of these strains. There were 2 strains in the third group and each, in turn, was shown to consist of a mixture of 2 strains, one resembling PR8 and the other resembling Weiss.

Attempts were made in 1955 to reisolate viruses from 8 of the throat washings and from 2 of the lung suspensions collected during 1943-1944. These attempts were successful in 3 instances, namely from one throat washing obtained during the height of the epidemic in December, 1943, and the other 2 from washings collected in February, 1944. These 3 fresh isolates included one from a 1944 washing the original isolation of which had yielded a strain that resembled PR8; these were studied after only 3 allantoic passages, by cross-neutralization tests in ovo along with other A strains that were isolated

in 1947 or later. The 3 newly isolated 1943-1944 strains were shown to be antigenically similar to each other and to Weiss, distinguishable from PR8, and entirely distinct from other A strains that were tested.

It is concluded that the postepidemic strains that resembled PR8 or were mixtures of such viruses with others resembling Weiss had been contaminated with the laboratory strain of PR8.

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SERUM GLUTAMIC OXALACETIC TRANSAMINASE IN EXPERIMENTAL TISSUE INJURY

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INTRODUCTION

RECENTLY Karmen[1] described a method of studying glutamic oxalacetic transaminase activity in the serum by coupling this reaction to the reduction of oxalacetate to malate by reduced diphosphopyridine nucleotide in the presence of malic dehydrogenase. LaDue[2, 3] used this method to investigate serum transaminase activity in a variety of clinical and experimental states. In both clinical and experimental myocardial infarction there was a significant increase in serum glutamic oxalacetic transaminase activity. Marked increase in serum enzyme activity was found in a variety of liver diseases as well as in experimental hepatitis.

The purpose of this study was to investigate serum glutamic oxalacetic transaminase levels in dogs subjected to either experimental infarction or ischemia of various organs.

METHOD

Dogs of mongrel variety were used and all animals were in apparent good health. Intravenous pentobarbital was used as an anesthetic agent. In all studies requiring thoracotomy, positive pressure oxygen was administered through an endotracheal catheter. All animals received 300,000 units of penicillin intramuscularly for three days following operation. Every precaution was taken to minimize tissue trauma of the surgery. Ribs were spread and not resected. Bleeding was almost always controlled by pressure with gauze sponges and the use of hemostats was assiduously avoided. Venous blood was drawn daily for five days after the operation or until the animal was sacrificed.

The tissues were examined by a competent pathologist (J. A. S.), and in cases where the extent or nature of the infarction was not readily apparent by direct inspection, the tissue was further studied by section and microscopic examination. In all experiments involving ischemia, microscopic studies were conducted to determine the presence or absence of necrosis. Microscopic sections of liver were studied in ten random specimens at post mortem.

The studies of ischemia were carried out with essentially the same technique. The artery leading to the organ (or part of the organ) was exposed and clamped off for varying periods of time. The absence of pulsations distal to the clamp and the pallor of the organ were used as the criteria by which ischemia was established. The myocardial ischemia experiments were conducted by the placing of a ligature about the main left coronary artery and the allowing of it to remain loosely in the chest with the ends buried subcutaneously. The ligatures were then exposed and traction applied to them causing ischemia, which was recorded by the marked elevation of S-T segments of the

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electrocardiogram. At the termination of the experiment, S-T segments returned promptly to normal, while no other abnormalities other than minor changes in electrical position were recorded.

The determinations of enzyme activity were done by the method of Karmen and LaDue.[1] All sera were tested in a laboratory where the temperature varied between 20 and 25° C. The reaction mixtures rapidly came to equilibrium with room temperature. When these temperatures were greater than 25° C., the enzyme activity in normal sera was greatly increased. There was an insignificant variability in activity when tests were conducted in the temperature range of 19 to 25° C. The sera were stored at 10° C. and tested usually within twenty-four hours, although occasionally a delay of seventy-two hours was encountered. The sera of sixty-three normal dogs were studied with a range of 8 to 39 units and mean of 21 units of activity (±6.2 S.E.).

RESULTS

A. *Infarction.*—

1. *Heart:* An attempt was made to produce small grossly visible infarcts as well as microscopic ones. In both instances there were infarcts which did not result in a significant elevation of serum transaminase activity. However, there was a rough correlation between the extent of the infarct and the peak of serum enzyme activity.

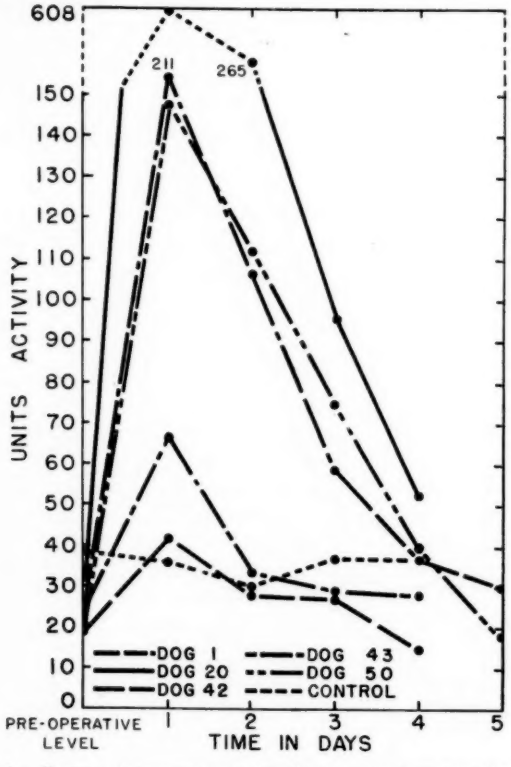

Fig. 1.—Serum transaminase levels in gross myocardial infarction.

The control procedure involved thoracotomy with incision of the pericardium which was sutured with catgut and the chest closed. The results of these procedures involving production of gross myocardial infarction are illustrated in Fig. 1.

Dog 1 had a ligature placed 1.5 cm. from the origin of the distalmost branch of anterior descending left coronary artery. The infarct was subendocardial and measured 3 by 2 by 0.5 cm. Dog 20 had its anterior descending branch ligated 3 cm. below the orifice of the main left coronary artery (below the first two lateral branches) and had two large areas of transmural infarction involving the left ventricle, septum, and the right ventricle. One area measured 3 by 5 by 0.3 cm., the other (over the right ventricle) 2 by 3 by 0.6 cm. A ligature was placed at a midway point on the first branch of the anterior descending branch in the experiment involving Dog 42. Numerous scattered areas of necrosis in an area 3.5 by 1.5 cm. were noted on gross inspection over the epicardial surface and extending through the wall of the myocardium to cover an area 1.5 cm. on the anterior papillary muscle. Microscopic sections confirmed the patchy characteristics of this infarct. Ligation of a similar vessel in Dog 43 was accomplished. The resultant gross area of infarct measured 1 by 2 cm. and again the infarcted areas were scattered. Interruption of the anterior descending branch of the left coronary artery midway in its course resulted in an area of transmural infarct measuring 2 by 3.5 by 0.3 cm. in Dog 50.

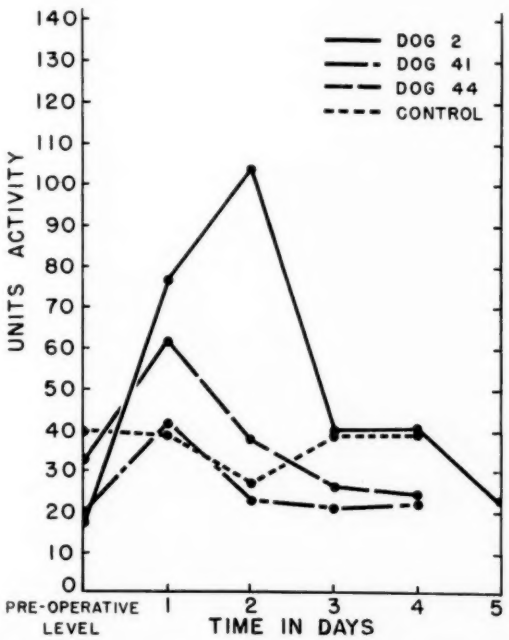

Fig. 2.—Serum transaminase levels in microscopic moycardial infarction.

In three animals (Dogs 2, 41, 44), microscopic areas of infarct were produced. The ligatures were placed at the apparent distal ends of main branches of the anterior descending branch. No macroscopic areas of necrosis were noted and the hearts were subjected to histologic examination. Sections taken near the area of ligature showed patchy areas of necrosis in a state of early organization consistent with organizing recent infarct. These results are summarized in Fig. 2.

2. *Lung*: The right lobar branches or the right pulmonary artery was ligated in six animals resulting in moderate to extensive pulmonary infarct with peak levels from 56 to 112 units of activity. The procedure of thoracotomy served as a control for these experiments.

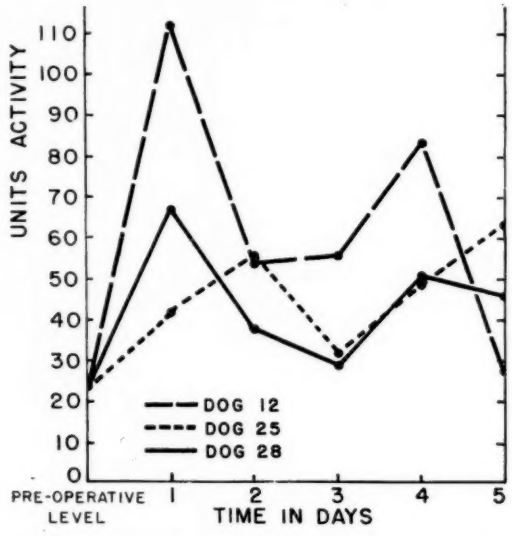

Fig. 3.—Serum transaminase levels following lobar artery ligations.

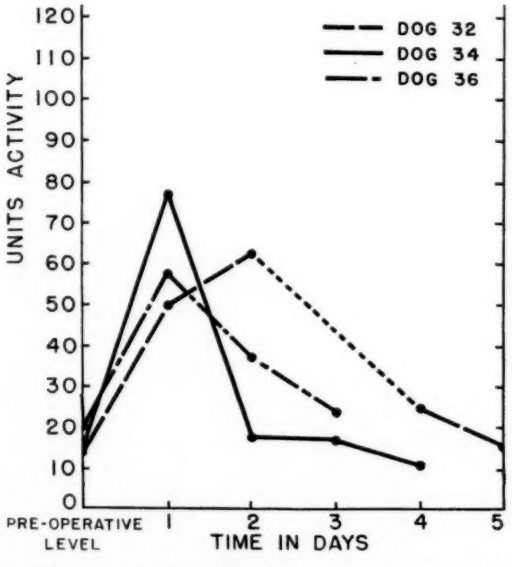

Fig. 4.—Serum transaminase levels following pulmonary artery ligations.

In Dog 25 the artery to the right lower lobe was ligated and post-mortem examination of this organ revealed multiple focal areas of infarct within an over-all area of 8 by 7 cm. Microscopic examination revealed many pulmonary infarcts with early organization. Dog 12 was subjected to ligation of the same vessel with patchy areas of necrosis throughout the right lower

lobe with organizing fibrinous pleuritis. In Dog 28 a similar vessel was ligated revealing diffuse nodular areas of infarct and atelectasis. The results are presented in graphic form in Fig. 3.

In Dogs 32, 34, and 36 the left main pulmonary artery was ligated with uniform gross involvement of the left middle lobe in all three. Right ventricular dilatation existed in all three specimens. Results are illustrated in Fig. 4.

3. *Kidney:* Interruption of the renal artery created irregularly shaped multiple infarcts (each less than 3 cm.) with peak levels of transaminase activity ranging from 114 to 149 units. Nephrectomy, which resulted in moderate amounts of necrosis of perirenal fat, caused a peak level of 58 units. The results are summarized in Fig. 5.

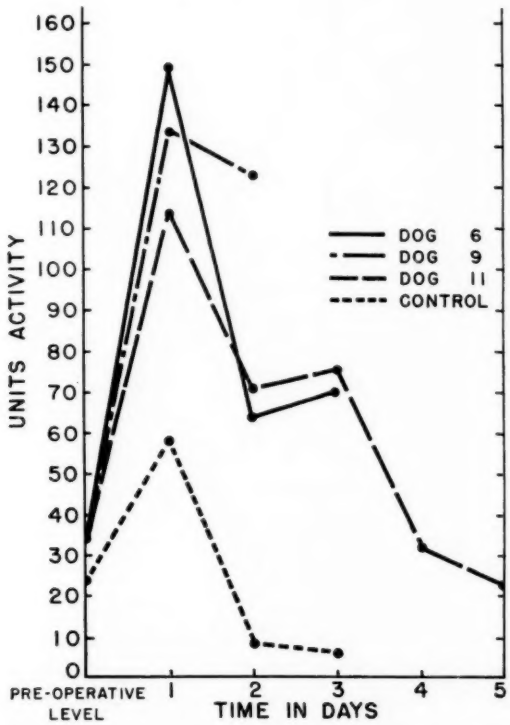

Fig. 5.—Serum transaminase levels in experimental renal infarctions.

4. *Spleen:* Splenectomy served as the control procedure and several of the vessels supplying the spleen were ligated in three other dogs. No attempt was made to dissect the artery from the corresponding vein and both vessels were ligated. In Dogs 9*B* and 18, four well demarcated infarcts varying in size from 1.5 to 4.0 cm. were observed. The spleen of Dog 10 revealed three areas, each approximately 2 by 4 cm. which had been infarcted. Peak levels ranged from 80 to 162 units. Fig. 6 illustrates the results.

5. *Intestines:* Infarcts of the bowel were varied in size and location with peak levels ranging from 79 to 221 units of activity. The control procedure involved opening of the abdominal wall and mechanically perforating the

sigmoid colon. Thereafter, the animal received no antibiotics. On the first postoperative day 5 ml. of fluid was aspirated from the peritoneum and culture revealed *Escherichia coli*. There was a peak serum transaminase level of 48 units.

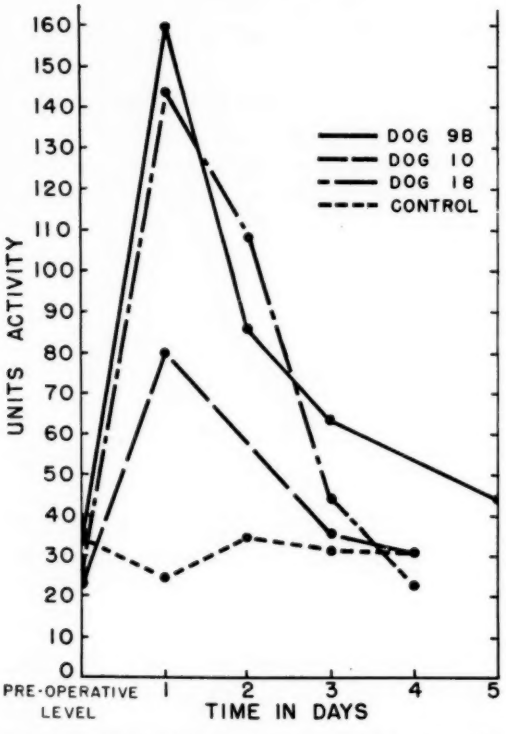

Fig. 6.—Serum transaminase levels in experimental splenic infarctions.

The operative procedure consisted of ligation of mesenteric arteries and veins. In Dog 5 there was an area 4 by 4 cm. on the antimesenteric border which was infarcted and was sealed off by omental adhesions. There was 300 ml. of cloudy serous fluid and microscopic examination confirmed the presence of ischemic necrosis of the bowel wall. In Dog 30 the infarct measured 3 cm. in length, involved the entire circumference of the gut, and was surrounded by extensive omental adhesions, while the gross pathology in Dog 8 indicated involvement of 14 cm. of small bowel with a distal loop of approximately the same length intussuscepted into the infarcted loop. These animals received streptomycin and penicillin for the entire postoperative period. The results are summarized in Fig. 7.

B. *Ischemia.*—The effects of this type of tissue injury were studied in seven animals, and in none of the sera tested were levels above the normal range encountered. In two animals the left renal artery was exposed and clamped off for periods of fifteen minutes in one and twenty minutes in the other, during which time no distal pulsations were observed and the organs were quite pale. The clamps were released after these periods and pulsatile

flow was promptly re-established. In other animals the vessels to an exposed loop of small bowel, spleen, and left main pulmonary artery were occluded for 15-minute periods.

Dog 70 was exposed to three five-minute periods of myocardial ischemia over a period of 45 minutes by a technique already outlined. Dog 71 had a total of nineteen minutes of ischemia over a thirty-minute period. The peak levels attained were 18 and 36 units, respectively. On microscopic examination both hearts revealed subsiding acute fibrinous epicarditis in the region of the suture and, in addition, Dog 70 had a small area of old perivascular fibrosis and organization with a focal interstitial myocarditis. Neither disclosed evidence of fresh infarct.

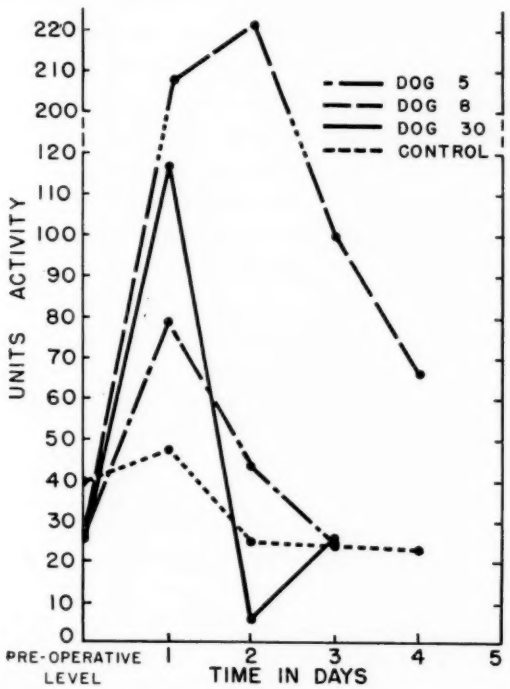

Fig. 7.—Serum transaminase levels following mesenteric vessel ligations.

DISCUSSION

It is evident in these studies that necrosis of many types of tissues results in elevation of the serum glutamic oxalacetic transaminase. As the amount of tissue rendered necrotic is reduced, the amount of enzyme appeared in the peripheral circulation is commensurately lessened and may fall within the normal range of activity of canine serum. This is also true of ischemia of short duration.

In all but one of the experiments involving production of a grossly visible myocardial infarct, there was a concomitant elevation of serum enzyme activity. Because of the patchy nature of small infarcts of the canine myocardium, it was deemed unrewarding to attempt dissection of these areas for

the purpose of comparing weights. The one gross infarct which produced only a very slight increase in serum activity measured 3.5 by 1.5 cm. over the epicardial surface and extended through the wall to cover an area of 1.5 cm. over the anterior papillary muscle (Dog 42). However, there was a rough correlation between serum enzyme level and the amount of necrotic myocardium in the other cases of gross infarct.

The characteristic feature of the microscopic myocardial infarcts was the variability of peak levels of enzyme activity from within normal range to distinctly elevated levels. It should be noted that in studying a few sera within the twenty-four-hour postinfarction period, and trying to correlate peak levels with amount of tissue involved, one ignores such factors as rate and duration of release of the enzyme as well as degradation or excretion.

Great emphasis was placed on minimizing the tissue trauma of surgery. It is felt that this factor explains the difference in postoperative levels in this series as compared with that of Nydick.[4] Each one of the control procedures was repeated several times with no significant elevation of enzyme level, with the exception of nephrectomy. In all of the ischemia experiments which involved an operative procedure and a period of ischemia, no rise in serum transaminase was noted in specimens taken for three subsequent days. Nevertheless, in all of the procedures there are obviously tissue trauma, inflammatory changes around the site of infarct, usually including pleura and peritoneum, as well as some degree of secondary infection of the superficial layers of the surgical wound. These factors may explain the elevation to fifty-eight units of activity after nephrectomy, which was always accompanied by some degree of necrosis of the perinephric fat. Although pericarditis followed every incision of the pericardium, there was no elevation in the control procedures, and this was true for several specimens taken from animals following the development of talc pericarditis. Pathologic study of liver in ten animals selected at random revealed no abnormalities.

In all but three of the experiments, the peak levels were achieved within twenty-four hours. Thereafter, most of the levels of activity fell rapidly and reached the normal range by the fourth day. In all of the experiments involving ligation of the lobar branch of the pulmonary artery there was a secondary rise in enzyme level in the fifth or sixth postoperative day. This is contrasted with ligation of the pulmonary artery where no such secondary rise is noted. The reason for the late rise is unknown.

Ischemia of various organs resulted in no abnormal elevation of serum glutamic oxalacetic transaminase activity in the peripheral blood, although it should be stressed that the ischemic state was of very short duration and did not produce gross or microscopic necrosis.

Transaminases are ubiquitous in their intracellular distribution throughout the order of mammals as well as lower forms of life. They may be associated with the secretory granules that have been isolated from saline extracts of liver and pancreas. These enzyme systems represent an important

mechanism in the synthesis and deamination of alanine and aspartic and glutamic acids, and thereby represent a method of integrating protein and carbohydrate metabolism. These enzymes are but one of a host of intracellular enzyme systems.

Other workers[4, 5] have recently reported analyses of saline extracts of normal and infarcted myocardium. It is considered that the results of these tissue studies are too fraught with the possibility of error to be reported as representing "glutamic oxalacetic transaminase activity." Crude saline tissue extracts will give the same results whether malic dehydrogenase is added or omitted from the reactants. Although little oxidation of reduced diphosphopyridine acetate (DPN) takes place until the alpha-ketoglutarate is added, there is at least one other enzyme system, certainly present in these extracts, which would oxidize reduced DPN under precisely these conditions. Glutamic dehydrogenase present in heart muscle in the presence of a source of nitrogen will convert α-ketoglutarate to glutamate with the oxidation of reduced DPN.[6, 7] Therefore, any quantitative measure of glutamic oxalacetic transaminase activity of these crude extracts is exposed to this error.

It would seem plausible to translate these observations in terms of similar infarcts occurring in man. There is similarity between responses noted in experimental myocardial infarcts and those clinical cases as reported by LaDue. The finding of a gross transmural infarct with only a minimal increase in serum transaminase activity when tested with two samples in the immediate postoperative period of twenty-four hours has not been reported by other workers.

This study would indicate that increased serum enzyme activity is a manifestation of tissue damage which is not characteristic of only the heart or liver. Nevertheless these two organs do contain relatively large quantities of this enzyme and infarction gives high serum values. Whether tissue damage occurring in disease states of these other organs is capable of releasing significant amounts of enzyme into the serum is not known. The difference in enzyme content of the various tissues is not so great that this possibility is a very real one.

SUMMARY

1. Elevations of serum oxalacetic glutamic transaminase levels were noted following infarct involving kidney, spleen, heart, lungs, and bowel.

2. Minimal elevations, noted in two of the control procedures, were probably due to the trauma of surgery.

3. A rough correlation was observed between amount of tissue involved and peak enzyme levels in experimental myocardial infarctions. One instance was observed of a gross transmural infarct with only a very slight abnormal rise in serum enzyme activity.

4. Ischemia of short duration produced no increase in enzyme activity.

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THE UTILIZATION OF A SYNTHETIC SUBSTRATE (TAMe) TO MEASURE THE PLASMA PROTHROMBIN IN COAGULATION DISORDERS

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PREVIOUS publications from this laboratory have demonstrated that a synthetic substrate, p-Toluensulfonyl-l-arginine methyl ester (TAMe) is hydrolyzed by thrombin.[1, 2] Based on this observation, a method was devised to measure plasma prothrombin after its conversion to thrombin by thromboplastin and calcium.[3] The present communication will be concerned with clinical experience with the TAMe assay for plasma prothrombin, at the same time comparing it with more conventional one-stage[4] and two-stage[5] assays in a variety of coagulation disorders.

The variations produced in the method by deficiencies of labile factor (V, Ac-globulin), stable factor (VII, S.P.C.A.), and the presence of heparin were determined before the assay was used clinically. Following these preliminary studies, the method was then utilized in the study of clinical disorders of the coagulation mechanism.

MATERIALS AND METHODS

Prothrombin Assays.—All one-stage assays were performed by the method of Quick.[4]

The TAMe assays were performed by the method of Glueck, Sherry, and Troll.[3] As previously noted, thrombin hydrolyzes the synthetic substrate p-Toluensulfonyl-l-arginine methyl ester (TAMe).[1] However, the presence of a high concentration (0.04 M) of the synthetic substrate in the activation mixture consisting of plasma, thromboplastin, and calcium inhibits the activation of the plasma prothrombin.[2] Lesser amounts of TAMe (0.001 M) inhibit the rate of the activation of prothrombin to thrombin, but the thrombin so evolved is protected by the dilute TAMe from destruction by antithrombin for a 5- to 10-minute interval, thus eliminating the need for frequent sampling of the mixture.[3] These observations have been utilized in the TAMe assay of plasma prothrombin.

Technique of the Assay.—Blood is collected in 0.1 M sodium citrate, one volume of anticoagulant being utilized for each 9 volumes of blood. A minimum of 5 ml. is needed for the test. The blood is centrifuged and the plasma defibrinated with bovine thrombin.* To each 1.8 ml. of plasma is added 0.2 ml. of thrombin (20 units). The fibrin clot is removed with an applicator stick, and the plasma stands at room temperature for one-half hour in order to allow the plasma antithrombin to remove the thrombin employed for defibrination.

Blank.—To 0.2 ml. of 0.4 M TAMe† (M.W. 373) in Tris buffer‡ (0.1 M Tris (hydroxymethyl) amino-methane (M.W. 121) adjusted to pH 9. with 0.1 N HCl) in a 15 ml. centri-

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*Parke, Davis bovine thrombin stock solution, stored at 8°C., is stable for months. Ten milliliters of a mixture of equal parts of glycerine and saline is added to each ampule containing approximately 5,000 N.I.H. units of thrombin. The preparation is diluted 1 to 10 with water before use.

†H. & M. Chemical Co., Santa Monica, Calif.

‡Eastman Kodak.

fuge tube, add 0.9 ml. of buffer,* 0.3 ml. of thromboplastin,† 0.3 ml. of 0.02 M $CaCl_2$ in buffer, and 0.3 ml. of defibrinated plasma.‡ Incubate the tube for 30 minutes at 37° C. At the end of incubation, add 2 ml. of formaldehyde, adjusted to pH 9.5-9.6 with 1.0 N sodium hydroxide.

Test.—To 0.2 ml. of 0.009 M TAMe in buffer, add 0.7 ml. of buffer, 0.3 ml. of thromboplastin in buffer, 0.3 ml. of calcium in buffer, and 0.3 ml. of defibrinated plasma. Activate for 5 minutes at room temperature.§ Add 0.2 ml. of 0.4 M TAMe in buffer and incubate for 30 minutes at 37° C. Terminate the reaction with 2 ml. of formaldehyde.‖ Centrifuge both "blank" and "test" tube at 2,000 r.p.m. for 15 minutes. Transfer 3 ml. aliquots of the clear supernatant to 25 ml. Erlenmeyer flasks. Titration is performed with 0.05 M sodium hydroxide with use of a syringe-type microburette. Two-tenths of a milliliter of phenol red (0.1 per cent, diluted with water 1 to 10 before use) serves as the indicator.

Calculation.—The TAMe unit has been arbitrarily defined as that amount of thrombin which will release 1 μM of acid from TAMe in 10 minutes under the conditions of the test. The TAMe unit is approximately equivalent to the activity of one National Institute of Health clotting unit of thrombin.[1] The following equation is used to calculate the number of TAMe units in one milliliter of plasma: (titration of test in milliliter – titration of blank in milliliter) × 91.4.¶

Two-Stage Assay of Plasma Prothrombin.—The method of Ware and Seegers[5] was used with slight modifications, plasma prothrombin being expressed as per cent of normal concentration rather than absolute prothrombin units. Lyophilized normal human plasma diluted with saline served as the reference standard.** Fibrinogen,†† and beef serum Ac-g‡‡ were obtained from commercial sources.

Qualitative Test for Stable Factor (S.P.C.A., VII Deficiency).—Two preparations were employed as the source of stable factor. Preparation one was made by the method of Lewis and Ferguson.[7] The second preparation was obtained with slight modification from serum, by the method of Stefanini and Dameshek.[8] Five milliliters of blood was collected in 0.25 ml. of saline-rabbit brain thromboplastin extract. After coagulation had occurred, the tube was incubated at 37° C. for one hour. The tube was then centrifuged and 0.1 ml. of 0.1 N sodium citrate added to each 0.9 ml. of serum. The serum was then aged at 8° C. for 2 to 3 days, divided into small aliquots, and frozen at –20° C. Such serum was stable for months but could not be refrozen after thawing.

When the test was performed, 0.1 ml. (10 per cent) of either fraction was added to 0.9 ml. of plasma and the usual one-stage assay performed, correction being made in the control for the dilution factor. If the material was being used in the TAMe assay, 0.03 ml. (10 per cent) of the fraction was added to the plasma and blank, and the volume of buffer was reduced by this amount (0.03 ml.).

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*All subsequent references to buffer indicate 0.1 M Tris buffer, pH 9.

†Difco. prepared according to manufacturer's directions, but in Tris buffer.

‡If plasmas of very low prothrombin content are being assayed (10 per cent or less) 0.4 ml. of plasma can be added to both blank and test, and the volume of buffer be reduced by 0.1 ml., the other reagents remaining the same.

§Dog and rabbit plasma which contain larger amounts of Ac-g[6] should be activated for 3 instead of 5 minutes, since at 5 minutes the thrombin titer has already begun to fall.

‖After the addition of formalin, titrations can be delayed for 1 to 2 hours and performed collectively.

¶This factor is derived by multiplying 10/9 (anticoagulant) × 10/9 (defibrination) × 10/3 (aliquot of plasma used) × 4/3 (aliquot of reaction mixture titrated) × 1/3 (reduction to 10-minute period) × 50 (conversion of standard alkali to micromoles). For routine assays, consideration of the hematocrit in determination of the anticoagulant dilution has not been deemed necessary.[5] For more precise calculations especially where the hematocrit is abnormally high, the hematocrit should be taken into account in calculating the dilution factor.

**"Protrol," Knickerbocker Biological, N.Y.

††Armour fraction I.

‡‡Difco. The purity of the reagents was tested by measuring the clotting time of a mixture of 0.1 ml. beef serum Ac-g, 0.1 ml. of thromboplastin, 0.1 ml. of 0.02 M $CaCl_2$ and 0.1 ml. of prothrombin-free plasma adsorbed with 50 mg. of $BaCO_3$ per milliliter of plasma. The reagent was used only if the clotting time of the mixture exceeded 3 minutes.

Qualitative Test for Labile Factor (Ac-g, V) Deficiency.—Commercial beef serum Ac-g (see two-stage assay) was used as the source of the factor. It was added in excess (10 per cent) to the plasma under study, 1 volume of the reagent being added to each 9 volumes of plasma. The usual one-stage assay was then performed with proper correction being made in the control for the dilution factor. When beef serum Ac-g was used in the TAMe assay, 0.01 ml. (3 per cent) was added to both the "blank" and the "test" mixtures. This quantity sufficed in the latter method.

Residual Serum Prothrombin.—The method of Sussman, Cohen, and Gettler[9] with slight modification* was used for the one-stage assay. The two-stage assay of Graham, Langdell, and Brinkhous[10] was employed when indicated. When the TAMe assay was utilized, 0.3 ml. of serum was substituted for the plasma of the usual method.

The plasma prothrombin was first assayed by the desired method. Additional samples of blood of exactly 2 ml. volume were collected in each of four 1 by 10 cm. tubes. The tubes were immediately placed in the water bath at 37° C. Fifteen, 30, 45, and 60 minutes, respectively, after the blood had been drawn, the further conversion of prothrombin to thrombin was terminated by the addition of 0.2 ml. of 0.1 M sodium citrate. The formed clot was disrupted with stirring, the tubes centrifuged, and the serum obtained incubated for an additional 15 to 20 minutes to destroy the free thrombin remaining in the serum. The residual serum prothrombin was then determined by the desired method.

The Effect of Labile Factor, Stable Factor, and Heparin on the TAMe Assay of Plasma Prothrombin.—

Labile factor (Ac-g, factor V) deficiency: Ware and Seegers,[5] recognizing that the two-stage assay of plasma prothrombin was affected by a deficiency of labile factor, subsequently modified the two-stage method so that labile factor (beef serum Ac-g) was routinely added to the plasma under study. The TAMe assay is likewise affected by a deficiency of this factor. There is little change in the TAMe assay of plasma prothrombin until the one-stage method shows a marked prolongation in prothrombin clotting time. Citrated blood is an excellent preservative of labile factor.[11] On prolonged storage, however, labile factor disappears from such plasma. The factor is less stable in oxalate solution and is destroyed by heating in oxalate at 37° C.[12] Table I illustrates the role of labile factor deficiency on the TAMe assay. When oxalated plasma was made deficient in labile factor, either by prolonged storage at 8° C., or by heating at 37° C., no striking difference was noted until the one-stage assay had fallen to approximately 40 per cent of its initial value. Under these circumstances, the TAMe assay fell 10 per cent. When the blood had developed a gross deficiency of the factor (23 per cent of normal, one-stage method) the TAMe assay had fallen only 22 per cent from its initial value. When, however, a gross deficiency of labile factor had altered the TAMe assay of plasma prothrombin, the addition of 0.01 ml. of prothrombin-free beef serum Ac-g† to such plasma restored the prothrombin to its initial level. Larger amounts of Ac-g were no more effective than 0.01 ml., regardless of the degree of the deficiency of the factor. Thus, even on prolonged storage, prothrombin itself appeared unaltered. Similar observations have been made by Fahey and associates[11] employing a two-stage assay method. Since the present studies have shown that the TAMe assay is altered by a gross deficiency of labile factor, we have modified the initial method[3] by the

*Citrate was added to the serum at the completion of incubation, and the serum then warmed for an additional period (see below).

†Commercially available Difco preparation (see "Methods").

routine addition of 0.01 ml. beef serum Ac-g to the assay whenever a deficiency of labile factor is suspected. As will be subsequently demonstrated, this is particularly important when both labile and stable factors are deficient in the same plasma.

TABLE I. EFFECT OF LABILE FACTOR (Ac-g, FACTOR V) DEFICIENCY ON THE ONE-STAGE AND TAMe ASSAYS OF PLASMA PROTHROMBIN

SOURCE OF DEFICIENCY		ONE-STAGE ASSAY (% NORMAL CONCENTRATION)	TAMe ASSAY μ/ML. (NORMAL 53 ± 5 μ)
A. *Citrated blood bank plasma, stored 8° C.*			
Days stored	0	70	42*
	6	52	43
	13	48	41
	20	33	40
	37	56	39
	49	41	37
	80	26	34
	106	25	33
	121	23	30
	160	10	15† ml.; + 0.01 Ac-g, 44 μ/ml.
B. *Oxalate plasma 0.1 M stored 8° C.*			
Days stored	0	100	53
	1	100	53
	4	66	52
	7	35	49
	9	18	22
	11	10	12† + 0.01 AC-g, 52 μ/ml.
C. *Oxalate plasma 0.1 M, heated 37° C.*			
Hours heating	0	75	45
	1	65	45
	2	42	43
	3	38	37
	4	25	36
	6	10	7

Plasmas were rendered deficient in labile factor (Ac-g, factor V) by storage or heating. The plasmas were then assayed by the one-stage and TAMe assays.

*The TAMe assay of this plasma was slightly lower than usual due to the high concentration of citrate used as anticoagulant.

†TAMe assay on addition of 0.01 ml. beef serum Ac-g[5], the method being otherwise unaltered; 0.01 ml. of beef serum Ac-g is an optimum concentration.

Stable factor (S.P.C.A. convertin, factor VII) deficiency: Since the classic studies of Orwin[13] and Alexander and his group,[14] it is known that Dicumarol depresses not only plasma prothrombin but also an accessory factor, stable factor (convertin, S.P.C.A., factor VII) as well. The disorder is likewise seen in patients with liver disease, and rarer disorders* (Case 7, Table II) are

*The one-stage prothrombin time in reported patients with congenital S.P.C.A. deficiency is usually more prolonged than in the present case.[15, 16] The criteria for the diagnosis are as follows: A white man 43 years of age, with no familial history of hemorrhage, had noted hemorrhage with dental extraction since youth. There was no laboratory or clinical evidence of liver disease. Clotting time and bleeding time were normal. Prothrombin time, one-stage, ranged between 40 to 70 per cent of normal, when repeatedly checked in a reliable laboratory (Jewish Hospital) over a seven-year period. Ac-globulin deficiency was absent as shown by ability of patient's plasma to correct the prolonged prothrombin time of aged, stored Ac-globulin-deficient plasma and inability of adsorbed normal prothrombin-free and stable factor-free plasma to correct the defect in the patient's plasma.[8] Evidence for a deficiency of stable factor was: inability of patient's serum to shorten the prothrombin time of a mixture of 5 per cent normal plasma and 95 per cent prothrombin, and stable factor-free plasma[3]; shortening of patient's prothrombin time on addition of serum[3]; less effective shortening of the prothrombin time of Dicumarol plasma on addition of 10 per cent of the patient's serum or plasma to the Dicumarol plasma than noted with normal serum or plasma[16]; failure in prothrombin response to large doses of vitamin K_1 emulsion.[3]

occasionally encountered with a deficiency of prothrombin, stable factor, or both. Such a group of patients was utilized to study the effect of the deficiency of this factor on the TAMe assay (Table II). The factor was prepared by two methods, that of Lewis and Ferguson[7] and that of Stefanini and Dameshek.[8] The fractions were then added in excess (10 per cent solution) to the plasmas of these patients, which were then assayed by the one-stage and TAMe methods. Inspection of Table II reveals the shortening of the one-stage prothrombin time on the addition of an excess of these fractions to the patient's plasma. In patients with only a moderate deficiency of stable factor, the one-stage prothrombin clotting time often attained normal values (Table II). Stefanini and Dameshek[8] similarly noted restoration to normal values of plasma prothrombin when an excess of serum was added to plasmas with only a moderate deficiency of stable factor. They likewise observed shortening of the plasma prothrombin on the addition of an excess of serum even when no deficiency of stable factor was present.

TABLE II. THE EFFECT OF STABLE FACTOR (S.P.C.A., CONVERTIN, FACTOR VII) DEFICIENCY ON THE TAMe ASSAY OF PLASMA PROTHROMBIN

SOURCE OF DEFICIENCY	ONE-STAGE ASSAY (% NORMAL CONCENTRATION)	ONE-STAGE ASSAY + 10% STABLE FACTOR	TAMe μ/ML. (NORMAL 53 ± 5 μ)	TAMe μ/ML. + 10% STABLE FACTOR
A. *Addition of Convertin (Lewis and Ferguson[7])*				
Dicumarol	66	100	28	29
Cirrhosis	48	100	16	15
Dicumarol	38	84	24	24
Caeliac disease before vitamin K	< 5	21	2	4
Caeliac disease after vitamin K	35	49	10	11
B. *Preparation of Serum (Stefanini and Dameshek[8])*				
Dicumarol	70	100	56	54
Congenital (?) deficiency of stable factor	50	100	53	53
Post-partum bleeding	56	78	14	17
Dicumarol	45	70	27	28
Cirrhosis	40	100	35	36
Phenylindandione	34	38	5	4
Cirrhosis	33	54	23	21
Cirrhosis	30	53	24	21
Dicumarol	23	50	17	19
Dicumarol	18	23	2	3
Dicumarol	14	24	9	12
Congenital atresia of bile ducts	12	35	3	2

The plasma was first assayed by the one-stage assay of Quick. One one-hundredth of a milliliter (10 per cent) of either the preparation of Lewis and Ferguson[7] or serum S.P.C.A. of Stefanini and Dameshek[8] was then added. After correction for dilution, the plasma was reassayed. In the TAMe assay, 0.03 ml. (10 per cent) of the factor was added to the test mixture.

The TAMe assay, however, appears relatively insensitive to a deficiency of stable factor until the one-stage assay reaches approximately 20 per cent of normal concentration. Even under these circumstances the addition of either the Lewis and Ferguson preparation[7] or that of Stefanini and Dameshek[8] produces only a moderate increase in the yield of prothrombin as assayed by the TAMe method (Table II). The TAMe assay is inaccurate below

10 per cent of normal concentration of prothrombin. At these low levels of prothrombin it is difficult to judge the effect of an associated stable factor deficiency. In practice it is unnecessary to add stable factor to the TAMe assay, since it is so little affected by this deficiency (Table II). An exception to this statement is combined stable and labile factor deficiency. Under these circumstances optimum yields of prothrombin are obtained if both 0.01 ml. of beef serum Ac-g and 0.03 ml. of either preparation of stable factor[7, 8] are added to the TAMe assay system.

TABLE III. EFFECT OF HEPARIN IN VIVO ON THE TAMe ASSAY OF PLASMA PROTHROMBIN IN A DOG

HEPARIN	CLOTTING TIME (LEE, WHITE) (MINUTES)	ONE-STAGE (%)	TAMe (μ/ML.)
None	4	100	40
10 Minutes after heparin	No clot 3 hr.	21	31
60 Minutes	60	50	37
240 Minutes	9	100	41

The plasma prothrombin of a mongral dog weighing 11 kilograms was first assayed by the one-stage and TAMe assay. The clotting time was determined by a modified method of Lee and White.[17] Fifty milligrams of heparin were then given intravenously and the clotting times and prothrombin assays repeated as indicated.

Heparin and the TAMe assay: In vivo, heparin has little effect on the TAMe assay as shown in Table III. A dog weighing 11 kilograms was given 50 mg. of heparin intravenously. When the clotting time was longer than three hours, the TAMe assay had fallen from an initial value of 40 units per milliliter to 31 units. When the clotting time was one hour long, the TAMe assay was 37 units. Similar observations have been noted in patients receiving heparin. In a heparinized patient with a coagulation time of 30 minutes (initial value 5 minutes), the prothrombin time had fallen from an initial value of 100 per cent to 55 per cent of normal when measured by the one-stage method. The TAMe assay, however, remained at the initial value of 46 μ per milliliter. The in vitro effect of heparin, which is more complex,[18] is at present under study.

Clinical Experience with the TAMe Assay.—Following the studies of the variables affecting the TAMe assay of plasma prothrombin, the method was utilized in studying a group of patients with coagulation disorders, when necessary comparison being made between the method and standard procedures.

Labile factor (Ac-g, factor V) deficiency: A group of patients with labile factor deficiency was studied by the TAMe assay. The deficiency was associated with a variety of causes as shown in Table IV. Included were 8 patients with cirrhosis, 6 with hepatitis, a case of obstructive jaundice associated with congenital atresia of the bile ducts in a child, 7 cases of post-partum hemorrhage, 1 case of carcinoma of the stomach with hepatic metastases, and a child with labile factor deficiency of unknown etiology.

The plasmas were first assayed by the one-stage method of Quick.[4] The same assay was then repeated with the addition of 10 per cent beef serum Ac-g. The TAMe assays were likewise performed with and without the addition of 0.01 ml. (3 per cent) prothrombin-free beef serum Ac-g. Although the results were not as clear-cut as the defect produced with storage or heat (Table I), the TAMe assay of the plasma prothrombin of these patients was usually unaffected by the deficiency of labile factor until the one-stage assay of the plasma prothrombin had fallen to approximately 50 per cent of normal

TABLE IV. PLASMA PROTHROMBIN IN PATIENTS WITH LABILE FACTOR (Ac-g, FACTOR V) DEFICIENCY, STUDIED BY THE ONE-STAGE AND TAMe ASSAYS OF PLASMA PROTHROMBIN

CAUSE OF DEFICIENCY	ONE-STAGE ASSAY (% NORMAL CONCENTRATION)	ONE-STAGE ASSAY + 10% BEEF SERUM Ac-g (% NORMAL CONCENTRATION	TAMe ASSAY μ/ML. (NORMAL 53 ± 5)	TAMe ASSAY μ/ML. + 3% BEEF SERUM (Ac-g
Liver disease:	33	52	23	29*
Cirrhosis 8	35	79	25	29
Hepatitis 6	40	50	8	10*
	45	80	12	10
	45	100	29	43*
	45	75	16	17
	45	86	20	28
	47	70	31	31
	55	80	29	30
	56	80	11	12
	58	62	21	23
	68	82	11	11
	70	94	35	35
	80	100	37	34
Congenital atresia, bile ducts (child)	52	80	3	2*
Post-partum hemorrhage	<5	<5	29	43†
	20	45	29	33
	35	90	46	50
	43	100	44	50
	45	70	12	14
	50	78	14	20
	50	70	54	56
Carcinoma stomach, hepatic metastases	50	70	54	56
Labile factor deficiency, child—unknown etiology	50	80	36	38

*Combined Ac-g, S.P.C.A. deficiency.
†Fibrinogenopenia, labile-factor, stable-factor deficiency.

concentration.* When the one-stage assay ranged between 40 to 50 per cent of normal concentration, the addition of AC globulin caused a moderate but definite enhancement of prothrombin yield as assayed by TAMe. When the one-stage assay fell below 40 per cent normal concentration, the effect of the deficiency on the TAMe assay was more striking. This was particularly true

*Case 6, post partum hemorrhage, showed a greater increase than usual in the TAMe assay when Ac-g was added.

if both stable and labile factor deficiencies were present. Regardless of the degree of deficiency, 0.01 ml. of beef serum Ac-g completely activated the plasma prothrombin to optimum levels. Larger amounts were unnecessary for maximum yields of thrombin from prothrombin.

TABLE V. THE RESPONSE TO DICUMAROL THERAPY AS ASSAYED SIMULTANEOUSLY BY THE ONE-STAGE AND TAMe ASSAYS

	NO. PATIENTS	ONE-STAGE (DAYS)	TAMe (DAYS)	t*
Days required for plasma prothrombin to fall to 30 per cent ± 5 of pretreatment level	16	5.1 ± 3.2	7.8 ± 3.0	< 0.05
Days required for plasma prothrombin to rise to 30 per cent ± 5 above lowest level found on discontinuing Dicumarol	9	2.0 ± 1.4	5.7 ± 4.0	< 0.05
Days required for plasma prothrombin to rise to 70 per cent ± 5 of pretreatment level on discontinuing Dicumarol	8	4.0 ± 1.1	7.5 ± 3.1	< 0.02
Days required for plasma prothrombin to attain pretreatment level on discontinuing Dicumarol	9	8.6 ± 3.9	23 ± 8.3	< 0.01

*Possibility of occurrence being due to chance alone less than 1 in 20, 1 in 50, or 1 in 100.[19]

Response to Dicumarol as measured by the TAMe, one-stage, and two-stage methods: In a preliminary communication, a small group of patients receiving Dicumarol was studied by the TAMe and one-stage assay methods.[3] These observations have been extended in the present study to include an additional 16 patients. Although individual variation in response to Dicumarol is so well known as to require no comment, it was observed on initiation of therapy in all the patients studied that the fall in prothrombin as measured by TAMe was less rapid than that noted with the one-stage assay. When Dicumarol was discontinued, the differences were even more marked. The plasma prothrombin always showed a more rapid rise when the one-stage assay was utilized. The rise was more gradual when measured by the TAMe method. The data have been summarized in Table V. The number of days required for the plasma prothrombin to fall to fixed levels on initiation of Dicumarol therapy and to rise to fixed levels after discontinuing the drug has been compared, with use of the one-stage and TAMe methods. We have not studied the response induced by other cumarol derivatives, which may differ from this pattern.

The plasma prothrombin of an additional 5 patients receiving Dicumarol was assayed simultaneously by the one-stage, modified two-stage, and TAMe methods. Fig. 1, characteristic of all five patients, shows the close similarity of the two-stage and TAMe assay methods. When Dicumarol was discontinued in these five patients, as well as the larger group in whom the TAMe

and one-stage assays were compared, the TAMe method showed a characteristic lag in plasma prothrombin concentration after it had reached approximately 60 to 70 per cent of pretreatment levels (Table V, Fig. 1). This suggests that the regeneration of prothrombin, the only factor measured by TAMe[3] is depressed for a considerable period after the administration of Dicumarol, whereas stable factor (VII, S.P.C.A.) is regenerated more rapidly than prothrombin itself. These observations with the TAMe assay in patients treated with Dicumarol are similar to those obtained with the two-stage method by Olwin,[20] Hurn and co-authors,[21] Duff and associates,[22] Stefanini and Dameshek,[8] and by Orwin,[23] employing the "P & P" method.

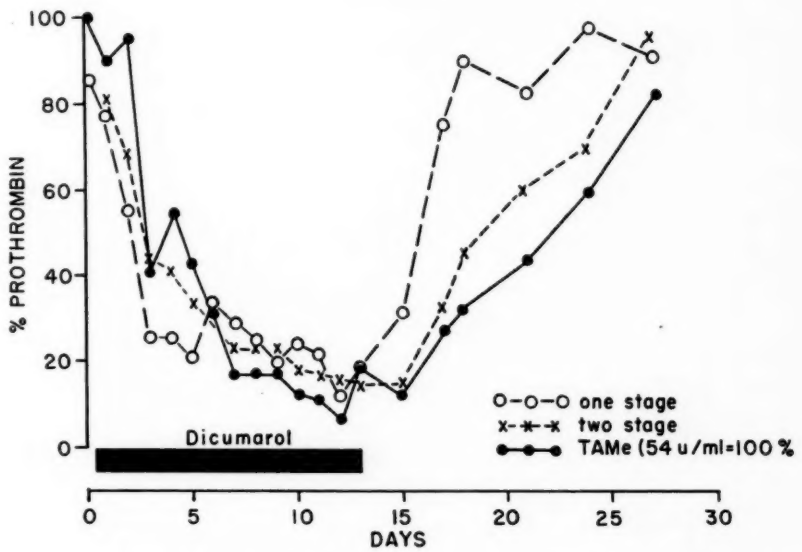

Fig. 1.—A comparison of the TAMe, one-stage, and modified two stage assays of plasma prothrombin in a patient receiving Dicumarol. The pretreatment assay of plasma prothrombin by the TAMe assay was 50 μ per milliliter (100 per cent). Ordinate, per cent prothrombin; abscissa, days of observation.

Vitamin K response as studied by the TAMe assay: The response to intravenous vitamin K_1 emulsion* was studied in dogs given Dicumarol. Four pairs of dogs were given Dicumarol, 8 mg. per kilogram on the first day, and 4 mg. per kilogram on the second. Subsequent Dicumarol dosage, determined by the one-stage method, was discontinued when the prothrombin had reached approximately 5 per cent of normal concentration. After several days at this level, Dog 1 was given 25 mg. of vitamin K_1 emulsion intravenously, while the second dog was allowed to recover spontaneously (Fig. 2, upper). In the dogs given Dicumarol but not given the vitamin, the return to pretreatment levels of prothrombin was always slower when measured by the TAMe assay than when determined by the one-stage method. These observations confirm those previously made on humans receiving Dicumarol (Table V). In the

*Mephyton, Merck & Company.

TABLE VI. THE RESPONSE TO VITAMIN K_1 EMULSION AS MEASURED BY THE ONE-STAGE AND TAMe ASSAYS OF PLASMA PROTHROMBIN IN PATIENTS WITH HEMORRHAGE ON DICUMAROL THERAPY

DISORDER	PRE-TREATMENT		DOSE VIT. K (MG.)	4 HOURS AFTER K		24 HOURS AFTER K		48 HOURS AFTER K		72 HOURS AFTER K		MORE THAN 72 HOURS AFTER K		COMMENT
	ONE-STAGE (%)	TAMe (μ/ML.*)		ONE-STAGE (%)	TAMe (μ/ML.)	ONE-STAGE (%)	TAMe (μ/ML.)	ONE-STAGE (%)	TAMe (μ/ML.)	ONE-STAGE (%)	TAMe (μ/ML.)	ONE-STAGE (%)	TAMe (μ/ML.)	
1. Cervical hemorrhage after cauterization, Dicumarol	16	6	100	33	19	80	24	77	33	100	30	100	44	TAMe assay required 8 days to reach pretreatment level. No hemorrhage after vitamin
2. Hematuria, coronary occlusion, Dicumarol	37	17	50	58	22	--	--	--	--	--	--	--	--	Dicumarol continued without futher hemorrhage
3. Hematuria, coronary occlusion, Dicumarol	12	< 5	50	52	7	35	7	48	8	78	14	78	21	Low level of prothrombin TAMe assay 21 μ/ml. 9 days after vitamin given, no hemorrhage after vitamin
4. Hematuria, thrombophlebitis, Dicumarol	23	3	50	--	--	100	4	--	--	100	12	--	--	Anticoagulant continued with no further hemorrhage
5. Hematuria, thrombophlebitis, "Hedulin"	23	2	50	38	3	--	--	--	--	--	--	--	--	Anticoagulant continued with no further hemorrhage
6. Hematuria, coronary occlusion, Dicumarol	30	2	25	--	--	70	23	60	--	--	--	--	--	Anticoagulant continued with no further hemorrhage

*Normal 53 ± 5 μ per milliliter.

vitamin K_1-treated dogs (Fig. 2, lower), the recovery period was shortened by the therapy. Even under these conditions, the TAMe assay always lagged behind the one-stage method. In the control animals not receiving the vitamin, a mean of 10.5 days was required for the one-stage plasma prothrombin to attain pretreatment levels, whereas an average of 17 days was required when the TAMe assay was employed. After the animals received the vitamin, a mean of only 1.7 days was required for the plasma prothrombin as measured by the one-stage method to reach initial levels, while a mean of 10 days elapsed before the TAMe assay reached this same point.

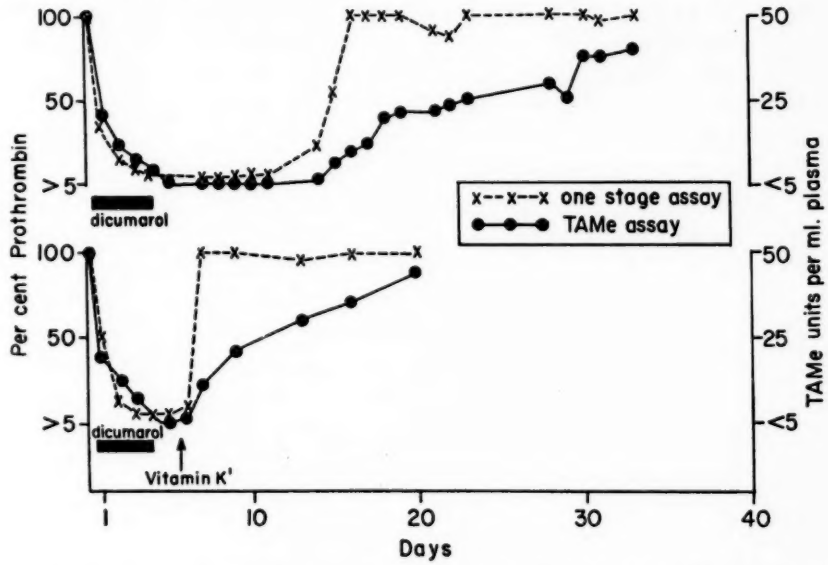

Fig. 2.—The response to vitamin K_1 emulsion in dogs receiving Dicumarol, measured simultaneously by the TAMe and one-stage assays. Upper figure, Dicumarol alone (bar); lower figure, Dicumarol followed by 25 mg. vitamin K_1 emulsion (arrow). The ordinate represents the per cent prothrombin, the abscissa the days of observation.

Because of the known variability from subject to subject in prothrombin response to Dicumarol, the experiment was repeated with a dog serving as its own control. The animal was first tested for Dicumarol response, as previously described. Six weeks after complete recovery, as determined by both assay methods, the dog was again treated with Dicumarol with an identical schedule of the drug and then given vitamin K. In a converse experiment, a second dog was given Dicumarol, followed by the vitamin, allowed to recover, and retested for Dicumarol response. Regardless of the experimental pattern employed, the response to Dicumarol and vitamin K therapy was always similar to that observed in the paired dogs.*

Table VI presents a group of patients with hemorrhage induced by Dicumarol.† Following a single intravenous dose of 25 to 100 mg. of vitamin K_1

*Mr. Rudy Donath offered valuable aid in the work with dogs.

†Three of the cases were the author's own, three were seen in consultation. However, the plasma prothrombin assays (both one-stage and TAMe) were all performed in the author's own laboratory on samples of plasma obtained both before and after the administration of vitamin K.

emulsion, the response was similar to that seen in dogs, that is, a more rapid rise toward normal levels of prothrombin when measured by the one-stage method, than that observed with the TAMe assay.

Hemorrhage associated with Dicumarol-induced prothrombin and stable factor deficiencies: Although it is conceded that hemorrhage is unlikely to occur in patients treated with Dicumarol when the prothrombin measured by the one-stage method is above 10 to 15 per cent of normal concentration, hemorrhage has often been observed when the prothrombin concentration is in the so-called "safe range."[24] Local causes often explain the defect; for example, undiagnosed duodenal ulcer, renal disease, or unsuspected malignancy.[25] In still other cases, the cause is unexplained. We have observed 6 patients with hemorrhage while on Dicumarol therapy in whom the prothrombin concentration when assayed by the one-stage method was within safe limits, with a range of 10 to 24 per cent one-stage method (Table VI). In 5 of the 6 patients, however, the TAMe assay revealed prothrombin of 6 units or less (normal 53 ± 5 μ per milliliter). In one patient renal hemorrhage was observed with a one-stage value of 24 per cent, and 32 per cent prothrombin (17 μ per milliliter) when assayed by TAMe.

For comparison, a larger group of 15 patients who showed no obvious hemorrhage while on continuous Dicumarol therapy was studied by the same techniques. One hundred and sixty-six plasmas were obtained from this group, with one-stage assays in the 10 to 30 per cent range. These same plasmas assayed by the TAMe method showed wide variation, from 2 to 34 μ per milliliter, with a mean of 11 μ per milliliter when assayed by the TAMe method. However, in only 21 of the one hundred and sixty-six plasmas studied was the TAMe assay less than 5 μ per milliliter.

The present series of patients showing hemorrhage with Dicumarol therapy is too small for statistical evaluation. The findings suggest, however, that hemorrhage at "safe" levels of prothrombin depression as measured by the one-stage method is more likely to occur when there is concurrent severe depression of both prothrombin and stable factor. Olwin[20] has made a somewhat similar observation employing the two-stage assay procedure in patients receiving Dicumarol.

The therapeutic management of the present series of patients was directed by the Quick one-stage assay method. In our experience this procedure is very satisfactory when there is careful laboratory supervision of the method and adequate normal control plasmas are utilized along with the specimen under study. The Quick one-stage method reflects changes of the all-over coagulability of the blood, indicating not only changes in prothrombin but stable factor as well. The insensitivity of the TAMe method to stable factor (S.P.C.A., convertin, factor VII) may be a disadvantage in its use for the control of Dicumarol therapy. Recently, however, Arscott, Koppel, and Olwin[26] who have had wide experience with the two-stage assay in Dicumarol therapy have reported favorably on the utilization of the TAMe method for this purpose. In the author's experience, the occasional determination of

plasma prothrombin with the TAMe assay was of value, however, particularly when long-term anticoagulant therapy was employed. If the TAMe assay reveals less than 5 μ per milliliter of plasma prothrombin particularly for several successive days, the dose of Dicumarol should be reduced regardless of the results obtained with the one-stage method in order to avoid hemorrhage.

Prothrombin and stable factor deficiencies other than those induced by Dicumarol: A group of 7 patients was observed with severe stable-factor and prothrombin deficiency (Table VII). It is of interest that in three of these patients the use of large amounts of antibiotics had apparently induced the acute deficiency.[8] In all the patients given vitamin K_1 (Mephyton) as in the Dicumarol-induced disorder, the one-stage method showed a more rapid rise toward pretreatment levels than did the TAMe assay. The dramatic improvement in the clinical status of the patients following the intravenous use of the vitamin must be ascribed to a rapidly rising titer of stable factor (S.P.C.A., factor VII). These observations suggest, however, that even after the plasma prothrombin as measured by the one-stage assay rises, such patients may benefit from an additional dose of the vitamin in order to accelerate the restoration of their "true" prothrombin to pretreatment levels.

Comparison of the TAMe, one-stage, and two-stage assays in patients with liver disease: Since the TAMe assay method is a two-stage procedure, it was postulated that the TAMe assay might be more closely related to the two-stage method than the one-stage method of Quick.[4] As noted previously, this proved true in patients treated with Dicumarol. It was felt that a more suitable group for study would be patients with liver disease or other coagulation disorders, in whom daily fluctuations in prothrombin and accessory factor changes are less striking than in patients receiving Dicumarol. Forty-five such plasmas were available for study. Twenty-six samples were obtained from patients with cirrhosis, 11 with hepatitis, 3 with obstructive jaundice, 2 with cardiac disease with marked congestive failure, and 1 each from a patient with metastatic gastric carcinoma of the liver, leukemic infiltration of the liver, and cardiac cirrhosis. Each plasma was assayed by the one-stage, modified two-stage, and TAMe methods. Fig. 3, left, shows the relationship between the TAMe and one-stage assay methods. Since the initial value of prothrombin by the TAMe assay was unknown, 100 per cent of normal was considered to be 53 units per milliliter, which we had previously found in normal subjects,[3] and the calculation deduced from this value. Considerable scatter about the slope can be observed when the TAMe method is plotted against the Quick one-stage assay method. Fig. 3, right, shows the TAMe assay plotted against the modified two-stage method of Ware and Seegers.[5] The close correlation and absence of scatter can be observed when the TAMe and two-stage methods are compared.

Residual serum prothrombin as measured by the TAMe, one-stage, and two-stage assay procedures: An additional method of comparison between the three methods was the measurement of the rate of disappearance of prothrombin from serum after coagulation had occurred.

TABLE VII. RESPONSE TO VITAMIN K_1 EMULSION IN PATIENTS WITH PROTHROMBIN AND CONVERTIN (S.P.C.A., STABLE FACTOR) DEFICIENCIES, AS MEASURED BY THE ONE-STAGE AND TAMe ASSAYS OF PLASMA PROTHROMBIN

DISORDER	PRE-TREATMENT		DOSE VIT. K (MG.)	4 HOURS AFTER K		24 HOURS AFTER K		48 HOURS AFTER K		72 HOURS AFTER K		MORE THAN 72 HOURS AFTER K		COMMENT
	ONE-STAGE (%)	TAMe (μ/ML.*)		ONE-STAGE (%)	TAMe (μ/ML.)	ONE-STAGE (%)	TAMe (μ/ML.)	ONE-STAGE (%)	TAMe (μ/ML.)	ONE-STAGE (%)	TAMe (μ/ML.)	ONE-STAGE (%)	TAMe (μ/ML.)	
1. Cardiac cirrhosis, severe congestive failure	33	26	100	48	28	61	26	54	32	--	--	54	26	One-stage, TAMe assay still low fifth day after vitamin. Patient also had Ac-g deficiency
2. Hematuria, indwelling catheter, continuous antibiotics for pyelitis	20	7	100	57	26	97	25	100	37	--	--	97	51	One month of Gantrisin, Aureomycin; streptomycin last 10 days of month. No hematuria 12 hours after vitamin given. TAMe assay normal eighth day
3. Hematuria, mild caeliac disease, child, pyelitis, antibiotics†	less than 5	1	50	--	--	35	11	--	--	80	37	90	45	Mild caeliac disease. Gantrisin for 4 days, for pyelitis, preceded hemorrhage. Given 250 ml. blood at onset of bleeding. 13 days required after vitamin, to reach normal levels with TAMe assay
4. Steatorrhea, idiopathic mild	76	40	100	82	42	91	40	--	--	--	--	85	38	Presenting symptom anemia. TAMe assay 38 μ/ml. fifteenth day after intravenous therapy. After 2 months of oral Mephyton (5 mg. daily), 52 μ/ml. TAMe assay

5. Congenital atresia, bile ducts, child	12	3	50	52	7	61	19	--	--	100	27	100	28	TAMe assay remained depressed (28 μ/ml.) 6 weeks after vitamin given
6. Hemorrhage, gastric, subtotal resection, continuous antibiotics	14	--	100	47	12	57	15	60	20	70	20	91	45	TAMe assay 45 μ/ml. 13 days after vitamin. Continuous streptomycin, Aureomycin, Gantrisin 10 days preceding and 8 days following surgery. Hemorrhage eighth postoperative day. No hemorrhage after vitamin
7. Dermatomyositis, and appendiceal abscess, child	38	--	25	70	24	100	40	--	--	100	45	—	—	Abscess associated with marked peritoneal hemorrhage at surgery

*Normal 53 ± 5 μ/ml.

†Dr. Eugene Lehay, Children's Hospital, kindly furnished plasmas from children.

Blood was collected in 0.1 M sodium citrate for the appropriate assay of plasma prothrombin. The remainder was collected in each of four test tubes and the serum prepared as described under "Methods." The serum prothrombin was then determined on the 15-, 30-, 45-, and 60-minute specimens. In the one-stage assay, the method of Sussman, Cohen, and Gettler[9] was utilized. For the two-stage assay the method of Graham, Langdell, and Brinkhous[10] was employed. The TAMe method was likewise used, as previously described.

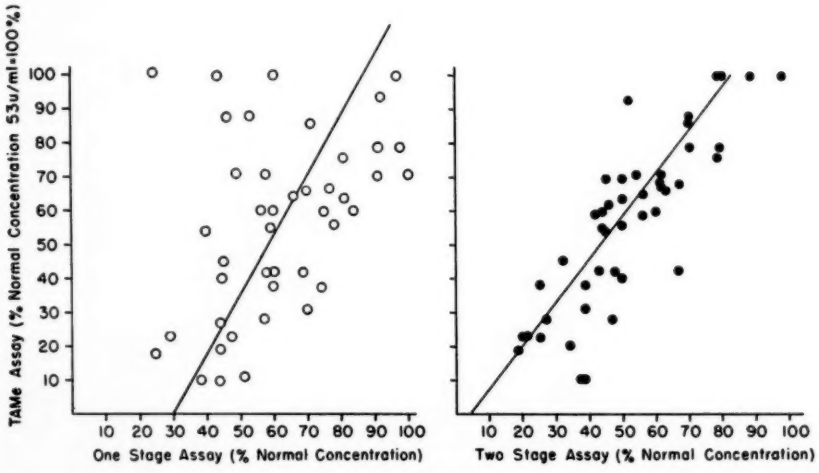

Fig. 3.—Plasma prothrombin in patients with liver disease assayed simultaneously by the one-stage, modified two-stage, and TAMe assays. Left, TAMe versus one-stage. Right, TAMe versus two-stage. The ordinate represents the TAMe assay, 53 μ per milliliter considered as 100 per cent. The abscissa represents the one-stage and two-stage assays. t value for left figure, 3.19 ($P = <.01$), right, 6.05 ($P = <.01$).[19]

Fourteen samples of blood from normal subjects were tested at the intervals described above, with the employment of the one-stage and TAMe assays. A hyper-reactive period in the serum, that is the 15- to 45-minute samples of serum, having a coagulation time the same as, or shorter than, the plasma, was usually seen with the one-stage assays, as previously noted by Brinkhous and co-workers.[27] However, this hyper-reactive period was never observed when the TAMe assay was utilized, the prothrombin concentration of the serum regularly falling once coagulation had occurred. Four of the sixteen samples were studied simultaneously by all three methods. Fig. 4 is a representative example. The two-stage and TAMe assays showed a downward fall in prothrombin concentration once the clot had formed. Sixty minutes after the drawing of blood in these normal subjects the mean value of the TAMe assay was 7 units (13 per cent) with a range of 5 to 25 μ (9 to 45 per cent). The mean value of the one-stage assay was 20 per cent, with a range of 10 to 60 per cent, while the four samples assayed by the two-stage method showed 16, 18, <10, and 20 per cent of initial concentration, respectively.

Hyperprothrombinemia and thrombosis: Nine cases with clinically apparent thrombophlebitis and 11 with myocardial infarction were studied on the first or second day of illness, before initiation of anticoagulant therapy. In no single instance was excessive prothrombin concentration observed when the plasma was studied by the TAMe assay.* The mean value of TAMe assay in the 20 patients was 50.5 μ per milliliter, with a range of 38 to 56 (normal 53 $\pm$ 5). The tendency to thrombosis in these patients appears to bear no relation to the level of plasma prothrombin.

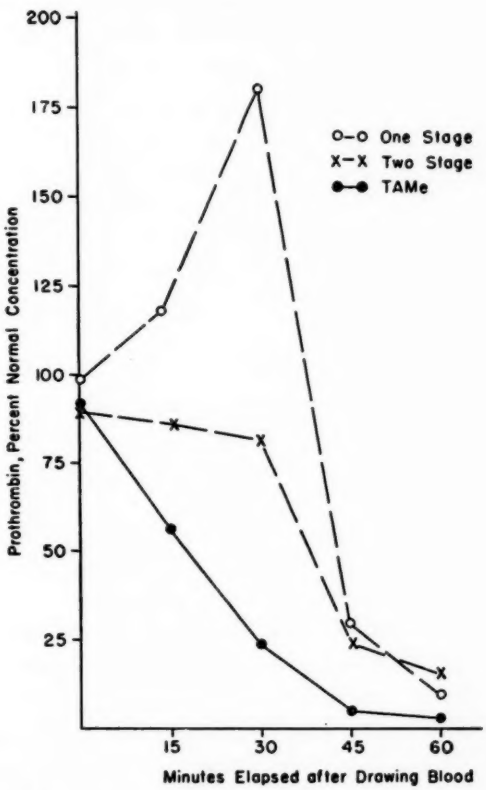

Fig. 4.—The residual serum prothrombin as measured by TAMe. The plasma prothrombin was first assayed by the one-stage, modified two-stage, and TAMe assays. Additional samples of blood (2 ml.) were collected in four test tubes. At the indicated times the reaction was stopped with sodium citrate. The serum prothrombin was then assayed by the one-stage, modified two-stage, and TAMe assays. (See text.)

DISCUSSION

The present studies have confirmed our preliminary observations that the TAMe assay is more closely allied to the two-stage assay methods than to the one-stage method of Quick.[4] This is not unexpected, since the TAMe assay is, in essence, a two-stage method. The findings in the present group of patients given Dicumarol closely parallel the extensive group studied by Olwin[20] and

*A smaller quantity than usual (0.2 ml. instead of 0.3 ml. of plasma) was utilized in these studies so that excess amounts of prothrombin beyond the range of sensitivity of the test system could be easily detected.[3]

others[8, 21-23] employing the two-stage assay. The similarity in response to vitamin K as measured by TAMe likewise parallels the findings of Bramble[28] employing the two-stage method. The absence of a hyper-reactive period in the serum after coagulation has occurred, when the TAMe and two-stage methods are compared with the one-stage method, again indicates their similarity. Finally, a direct comparison of all three methods in patients with liver disease indicates the close correlation which exists between the TAMe and two-stage methods.

The TAMe assay appears to offer certain advantages over methods depending on the coagulation of fibrinogen. One of the chief advantages of the method is its insensitivity to changes induced by moderately severe labile factor deficiency, marked stable factor deficiency or in vivo heparin. Because of its relative insensitivity to labile and stable factors, the present method is not well suited to their measurement. On the other hand, the fact that the TAMe assay measures only prothrombin offers a distinct advantage when multiple deficiencies in coagulation disorders occur, as shown in this communication in patients with cirrhosis or complex bleeding disorders.

Another advantage of the TAMe assay is the reproducibility of the method. Since TAMe is a chemical substance, its reactivity does not vary from preparation to preparation as does fibrinogen. We have obtained identical values for normal plasmas from four different lots of the material. The long, five-minute activation period of the method insures maximum conversion of prothrombin to thrombin and eliminates the necessity for the frequent sampling of the two-stage assay. The presence of dilute TAMe in the activation period protects the evolving thrombin from destruction by antithrombin. Therefore, dilution of the plasma, which serves to minimize antithrombin in the two-stage methods, is eliminated. Since previous studies have shown that the TAMe assay of plasma prothrombin is linear rather than parabolic,[3] the method is accurate in the 50 to 100 per cent range where the clotting methods are relatively insensitive.

Its close parallelism to the two-stage methods as shown in the present communication indicates its usefulness in the disorders of coagulation, which can be well studied by the former method. The technical simplicity of the TAMe assay, particularly in patients with multiple disorders in the coagulation mechanism, should serve as a useful tool for further studies in this group of patients.

SUMMARY

Studies have continued, employing a synthetic substrate, p-Toluensulfonyl-l-arginine methyl ester (TAMe), to measure plasma prothrombin. It has been observed that the TAMe assay is relatively unaffected by a deficiency of labile factor (V, Ac-g), and to a lesser extent by a deficiency of stable factor (VII, S.P.C.A.). When gross deficiencies of either factor are present, the addition of a fixed amount of the factors to the plasma will completely correct the deficiencies.

The present studies illustrate the close similarity of the TAMe and two-stage assays in response to Dicumarol, vitamin K therapy, and residual serum prothrombin. Direct comparison of all three methods in patients with stabilized liver disease emphasize the close similarity of the TAMe and two-stage assay methods. Because of this latter observation, and because of the reproducibility and simplicity of the method, the TAMe assay serves as a useful tool in the study of complex coagulation disorders.

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METABOLIC ALTERATIONS IN SURGICAL PATIENTS

IX. The Influence of Intravenously Administered Fat Emulsions on Nitrogen Balance in Postoperative Patients

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PREVIOUSLY it has been stressed[1-3] that the nitrogen deficit which follows many types of surgical trauma (subtotal gastrectomy, appendectomy, cholecystectomy, or combined abdominal perineal resection) is largely the result of the "starvation" regimen commonly employed during the postoperative period rather than the operative procedure per se. This regimen usually consists of two to three liters of a five or ten per cent hexose solution daily, with or without salt, and affords only 400 to 1,200 calories. By the providing of both nitrogen and calories during the postoperative period it has been found that the nitrogen deficit could be significantly reduced.[1-3] In recent years fat emulsions that can be given intravenously have been improved so that they are now reasonably well tolerated by both animals[4, 5] and man.[6-9] A fat emulsion, because of its high caloric content per unit volume, would be an excellent source of calories for a patient in whom it was necessary to provide all nutrition by the parenteral route (e.g., 600 ml. of a 15 per cent fat emulsion* containing 4.2 per cent carbohydrate yields approximately 900 calories). In order that it might be determined whether the fortification of an intravenous regimen, consisting of vitamins, protein hydrolysates, and hexoses with fat emulsions, would result in a further decrease in the postoperative nitrogen deficit the following studies were undertaken.

METHODS

The patients studied were admitted to the Metabolic Division of the University Hospitals of Cleveland where they were given diets which were analyzed for nitrogen, sodium, potassium, and chloride. All fluids which were used for intravenous alimentation were similarly analyzed. The nitrogen was determined by macro-Kjeldahl procedure, sodium, and potassium with use of a flame photometer with an internal standard and chloride by the method described by Van Slyke and Peters.[10] Urine samples and all other excreta vomitus, gastrointestinal suction, or sputum) were analyzed daily while the stool samples were pooled into periods of three to five days. The details used in carrying out the metabolic balance study has been described earlier.[11] A brief clinical discussion of the patients, when significant, will be given in the results.

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RESULTS

The data obtained from two patients (M. S. and J. B.) who each had an inoperable carcinoma of the esophagus are shown in Fig. 1. The metabolic balance results in these and all subsequent charts are plotted with the daily nitrogen intake being measured from the zero line upward. The total daily output of nitrogen from all excreta is plotted from the top of the intake column downward. Positive nitrogen balance is represented as a white area above the zero line and a negative nitrogen balance by the solid black areas below the zero line. Oral and intravenous intakes are indicated by the difference in shading of the columns. It should be noted that for three of the five days that M. S. was receiving fat emulsions he was in positive nitrogen

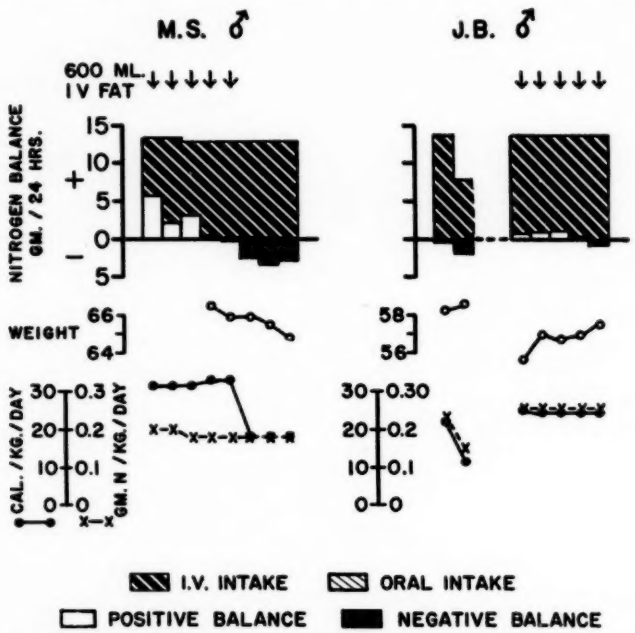

Fig. 1.—Effects of adding a fat emulsion to an intravenous regimen given to two patients with cancer of the esophagus.

balance and for the other two days he was either in equilibrium or showed a slight negative balance. On the other hand, during the next three days, while his nitrogen intake was held constant and his caloric intake was reduced, by our not giving the fat emulsion, he showed daily nitrogen deficits of from 2.5 to 3.5 Gm. per day. Patient J. B. was studied for two days before receiving the fat emulsions; then the investigation was interrupted for five days because of diagnostic studies. Following this period the full intravenous regimen was resumed. J. B. was essentially in nitrogen equilibrium during the time he was maintained on an intravenous regimen consisting of amino acids, hexose, and fat. Thus, these two nonoperated patients with cancer demonstrate nitrogen equilibrium or positive balance during the period of complete parenteral alimentation.

The fact that the administration of 2,100 calories, in the form of fat and fructose, cannot abolish the nitrogen deficit (unless adequate amounts of nitrogen are also given) is evident by the data presented in Fig. 2. Patient W. B., a 61-year-old man, had a subtotal gastric resection performed because of ulceration at the pyloric end of the stomach. He received 600 ml. of fat intravenously from the second preoperative day until the end of the study. From the day of operation until the third postoperative day he did not receive any protein hydrolysate but was given only carbohydrate and fat emulsions intravenously. During this time he showed a three-day cumulative nitrogen deficit of approximately 20 Gm. The next two days he received nitrogen, hexose, and fat intravenously, and showed a good positive balance on the first day and approximately the same degree of nitrogen deficit the next day which resulted in a cumulative nitrogen deficit of about 0.5 Gm. for this two-day period.

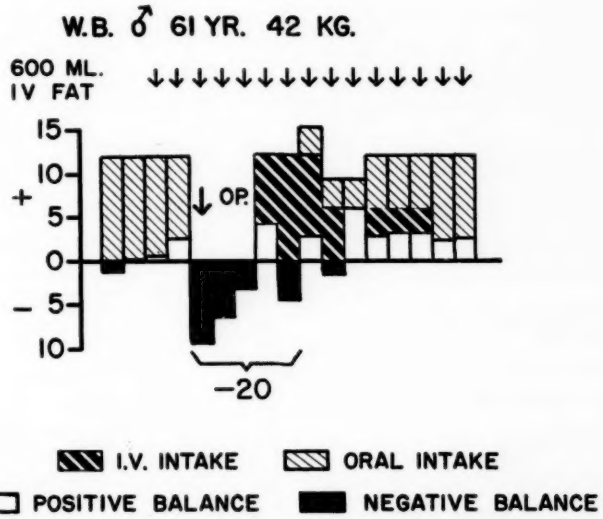

Fig. 2.—Effect of administering a fat emulsion to a patient during a nitrogen-free regimen.

In order that a comparison might be made with the nitrogen balance data of the different patients studied, a period consisting of the day of operation plus the first four postoperative days was selected since this represented a period when they received little or no oral nutrients and when the "stress" of operation was maximal. The data obtained from two patients who had a subtotal gastrectomy performed for peptic ulceration are presented in Fig. 3. Both individuals were maintained exclusively by intravenously administered nutrients for several days preoperatively and postoperatively until the fifth and seventh days. Patient J. C. showed a five-day postoperative nitrogen deficit of 13.5 Gm., and A. S. showed a nitrogen deficit of 12.3 Gm. during a similar period. It should be noted that when J. C. went on an oral diet he would not voluntarily consume a sufficient amount of nutrients during the

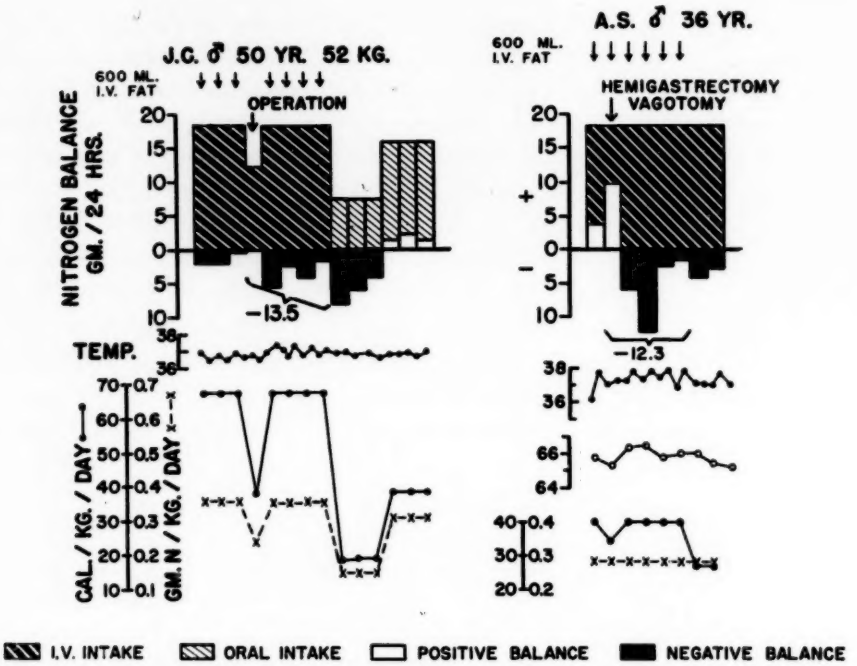

Fig. 3.—Nitrogen balance data on two surgical patients whose regimen as supplemented with fat emulsion.

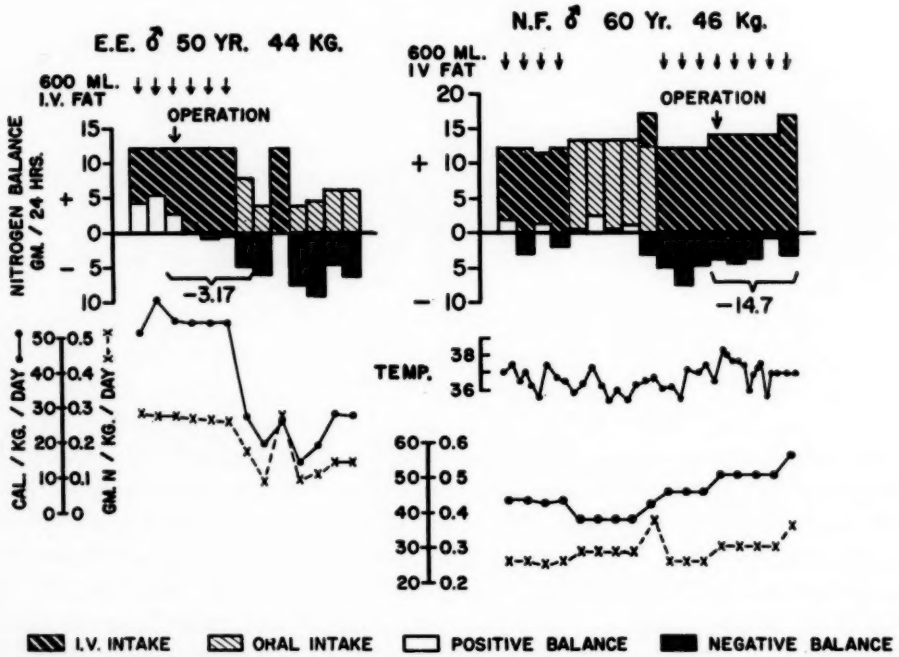

Fig. 4.—Nitrogen balance data on two surgical patients whose regimen was supplemented with a fat emulsion.

fifth, sixth, and seventh postoperative days to meet his caloric and nitrogen needs and, hence, exhibited a greater nitrogen deficit than noted during the previous five-day period.

Patients E. E. and N. F. (Fig. 4) both had subtotal gastric resections performed for malignancy of the stomach. Patient E. E. was small in stature (weight 46 kilograms) and consequently received a larger caloric intake per kilogram of body weight than N. F. During the five-day study periods, E. E. and N. F. showed cumulative nitrogen deficits of 3.17 Gm. and 14.6 Gm., respectively. The nitrogen balance data for E. E. again demonstrates that in the early postoperative period a nitrogen deficit may occur because the patient will not take orally sufficient amounts of the prescribed diet.

DISCUSSION

One of the problems of intravenous alimentation when use is made of carbohydrate solutions and protein hydrolysates only is that it is quite difficult to give the patient more than about 1,500 to 1,800 calories per day without the use of alcohol or a hypertonic (20 to 25 per cent) hexose solution. Such a nutritional regimen can be greatly fortified in regard to calories by use of a fat emulsion. The fat emulsions have the following advantages: a high caloric content in a small volume of fluid (approximately 900 calories per 600 ml.); the fat exerts very little osmotic effect and there is no urinary spillage; both fat and carbohydrate, by providing calories, allow more of the administered nitrogen to be utilized for tissue fabrication. In the case of a person of slight build it is possible to meet the caloric (30 to 60 calories per kilogram per day) and nitrogen (0.25 to 0.45 Gm. per kilogram per day) needs by administering only protein hydrolysates and hexose solutions. But in the case of persons of normal size and weight or larger, the volume of nutrients which must be administered to meet the caloric requirements is excessive unless the high calorie fat emulsions are employed.

The effect of caloric and nitrogen intake on nitrogen balance has been studied by both Benditt and associates[12, 13] and Calloway and Spector.[14] The latter investigators reviewed recent data concerning the influence of caloric and nitrogen intake on nitrogen balance in healthy young men. Individuals maintained on a zero caloric and nitrogen intake showed a nitrogen deficit of approximately 12 Gm. per day. On increasing only the caloric intake they noted a decrease in the daily nitrogen deficit. When the caloric intake reached 750 calories per day, a nitrogen deficit of 7 Gm. was found. On a nitrogen-free diet, a progressive increase of the caloric intake to 2,300 calories caused no further reduction in the nitrogen deficit. This indicates that on a nitrogen-free regimen the maximum effect of calories in reducing the nitrogen deficit is reached at an intake of approximately 750 calories and additional caloric supplementation has little or no effect in sparing nitrogen.

Calloway and Spector also reviewed the effect of adding calories to the diet of normal adults who were receiving nitrogen. On a nitrogen intake of between 7 to 8 Gm., with a caloric intake of only 400 to 600 calories per day,

subjects showed nitrogen deficits similar to those in a person ingesting no nitrogen and receiving only 400 calories. Thus, under these conditions (moderate nitrogen intake coupled with a deficient caloric intake) the nitrogen ingested was of little benefit for tissue fabrication since it was probably being used for energy purposes. By increasing the caloric intake to 900 calories per day and keeping the nitrogen intake approximately the same (7 to 8 Gm.), they noted a reduction in the nitrogen deficit, and by further increasing only the caloric intake to around 1,600 calories per day they found that the negative nitrogen balance fell to about 1.8 to 2.4 Gm. per day. This degree of negative nitrogen balance remained even if the caloric intake was increased to 2,300 calories per day, with the nitrogen remaining at approximately 7 to 8 Gm. per day. However, nitrogen equilibrium could be attained by feeding 8.5 Gm. of nitrogen and 3,355 calories per day. Calloway and Spector emphasized in the case of adult men that either the nitrogen intake or the caloric content of the diet may be the limiting factor in obtaining nitrogen equilibrium.

Recently, two reports concerning the administration of fat emulsions to surgical patients have appeared. Van Itallie, Moore, Geyer, and Stare[9] have reported that a normal individual who was maintained on a marginal nutritional intake showed decreases in both the nitrogen and potassium deficits after receiving fat emulsions intravenously. On the other hand, when fat emulsions were given intravenously to both a normal individual (maintained on a very low nitrogen and caloric intake) and to two postoperative patients (receiving no nitrogen), no decrease in the nitrogen deficit was noted. Similar results were reported by Shingleton, Anlyan, Salem, and Saunders[15] who maintained patients postoperatively on only intravenously administered sugar solutions and fat. Fig. 2 illustrates the nitrogen balance data obtained from a patient, in the present study, who received only hexose solutions and a fat emulsion on the day of operation and during the first two postoperative days. No retention of nitrogen occurred until nitrogen was added to the regimen. During these three days of the study he showed a cumulative nitrogen deficit of approximately 20 Gm., which was greater than that shown by the other subtotal gastrectomized patients (receiving nitrogen, carbohydrate, and fat) during the entire five-day study period (Figs. 3 and 4). These results are in accord with the observations of both Benditt and associates[12, 13] and Calloway and Spector,[14] who have shown that the caloric supplementation on a nitrogen-free regimen has little or no effect in reducing the nitrogen deficit.

It has been demonstrated that following various types of trauma, both patients and animals go into a period where they develop large nitrogen deficits. This has been considered by some workers to be one of the characteristic findings of the "stress" reaction. However, patients (Figs. 3 and 4) who received good intakes of both nitrogen and calories following a major surgical procedure (subtotal gastrectomy) did not show this large nitrogen deficit. But such a nitrogen wasting does occur in postoperative patients who are getting insufficient amounts of either nitrogen or calories, or both. In an

earlier investigation[1] we reported that a group of partially gastrectomized patients maintained on only a 5 or 10 per cent hexose solution without nitrogen showed a five-day average cumulative nitrogen deficit of 60 Gm. By increasing the caloric (1,200 to 1,600 calories) and nitrogen (6 to 9 Gm.) intake, we found that a comparable group of operated patients showed an average five-day cumulative nitrogen deficit of 30 to 35 Gm., and in the present study the patients who received the increased caloric supplementation showed five-day nitrogen deficits of around 3 to 14 Gm. The large nitrogen deficits following surgical trauma of the soft tissue thus seem to be mainly the result of poor postoperative nutrition rather than due to the "stress" reaction.

SUMMARY

The effect of administering fat emulsions intravenously to surgical patients who were being maintained on protein hydrolysates and hexose solutions was studied. Two patients with cancer of the esophagus treated in this manner showed either a decrease in their negative nitrogen balance or a positive balance. Four patients who had a subtotal gastrectomy and were maintained by intravenously administered protein hydrolysates, hexoses, and fat emulsions showed a five-day (day of operation plus the first four postoperative days) cumulative nitrogen deficit of 3 to 14 Gm. The effect of intravenously administered fat in decreasing the nitrogen deficit of these postoperative patients is believed not to be a special attribute of the fat but rather to be due to the additional caloric supplementation. The intravenously administered fat emulsion increased the caloric intake in these patients, who received adequate amounts of nitrogen and moderate amounts of calories, in the form of hexose, to such an extent that there was improved utilization of the protein hydrolysate, which resulted in a decreased nitrogen deficit.

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STUDIES ON THE ANTICOAGULANT PHENINDIONE

IV. Its Multiphasic Effect on the Coagulation Mechanism

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ALTHOUGH anticoagulants with a prothrombopenic effect have been used for over a decade, they are still the subject of debate concerning their effectiveness. Doubt arises from conflicting opinion over not only what level of prothrombopenia constitutes a therapeutic level, but also, indeed, whether any anticoagulant effect is achieved short of production of a hemorrhagic diathesis. Statistical analysis of large groups of treated patients leaves little doubt that some protection from thrombotic conditions can be achieved, but it is apparent that intravascular thrombosis can occur even when a therapeutic level of prothrombin time exists. Prevention of experimental thrombosis in animals has been observed,[1] and indeed recanalization of existing experimental thrombosis in treated animals can be demonstrated,[2] but usually hypoprothrombinemia far exceeding that possible in man was employed. Moreover, the difference between the natural concentrations of individual clotting factors in different species makes the results difficult to interpret in terms of what the therapeutic effect should be in man.

More precise knowledge of the effect of these preparations has developed sporadically over the past few years. For example it has been known for some time that Dicumarol affected not only prothrombin but also another factor which is intimately connected with the conversion of prothrombin to thrombin. This factor first described as the conversion factor of Dicumarol plasma by Owen and Bollman[3] has been further delineated under the names cothromboplastin, pro-SPCA, proconvertin, stable factor, and factor VII by different authors. In this paper it will be referred to as proconvertin. Most investigators feel that the prolongation of the one-stage prothrombin time by Dicumarol reflects mostly changes in proconvertin rather than changes in prothrombin. Biggs and Douglas[4] feel that the prothrombin time is in fact a measure of this factor rather than of prothrombin, because in congenital deficiency of proconvertin the prothrombin time is greatly prolonged while in isolated deficiency of prothrombin they noted relatively slight change in the one-stage prothrombin time compared to the marked reduction of prothrombin recorded by the two-stage test. Walker and Hunter[5] observed predominantly a deficiency of proconvertin in patients receiving phenindione.

More recently it has been suspected that a third factor which is concerned with the evolution of thromboplastin is affected by Dicumarol. Using the

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thromboplastin generation test, Walker and Hunter[6] noted that the serum of patients receiving Dicumarol generated thromboplastin poorly even though the prothrombin time of the patient was not greatly prolonged. Fluckiger, Duckert, and Koller[7] noted the same phenomenon and felt that a factor was missing which they termed "factor X." Sise, Adamis, and Kimball[8] were able by mixing experiments to show that a deficiency of plasma thromboplastin component (PTC) existed in patients who had received phenindione for long-term anticoagulant therapy. This factor is required for the normal elaboration of plasma thromboplastin along with platelets, antihemophilic globulin, and plasma thromboplastin antecedent. Congenital deficiency of PTC is associated with a hemorrhagic diathesis which is identical clinically to hemophilia.

It is the purpose of this paper to present data relating to the coagulation factors affected by phenindione. The results indicate that the relationship of the three aforesaid coagulation factors show chronological differences which have important bearing on the anticoagulant effect but which are not reflected by the one-stage prothrombin time. It is probable that a deficiency of prothrombin rather than proconvertin or PTC is most clearly related to an anticoagulant effect.

METHODS AND MATERIALS

Individuals from both a hospitalized and ambulatory group of patients who were receiving phenindione therapeutically[9, 10] were selected for study on the basis of prothrombin time and duration of treatment. One-stage prothrombin times were done by the method of Quick with use of dried rabbit brain thromboplastin (Difco) and calcium chloride 0.1 M. Fresh human plasma adsorbed with $BaSO_4$ 50 mg. per milliliter, was used to make up dilution curves for expressing one-stage prothrombin activity in per cent of normal. The test for proconvertin was done by Adamis' modification of Owren's method.[11] Prothrombin was measured by the two-stage method of Ware and Seegers.[12] AC globulin was measured by the shortening of the prothrombin clotting time of oxalated plasma made AC globulin-free by freezing for several weeks and then incubating at 37° C. for 24 hours. Glass clotting times were done by a modification of the Lee White technique described by Moloney[13] in which the normal values lie between 7 and 14 minutes.

The thromboplastin generation test on subjects receiving phenindione was performed by the method of Biggs and Douglas[14] with the following modifications: sodium oxalate 0.1 M one part to nine parts of whole blood was used in place of citrate, $BaSO_4$ 10 mg. per milliliter of plasma was used as the adsorbing agent in place of aluminum hydroxide suspension, and serum was oxalated after one-hour incubation at 37° C. and then further incubated another hour. The minimal clotting time in this test with normals developed in 4 to 13 minutes of incubation and was 6 to 13 seconds.

Sodium oxalate 0.1 M one part to nine parts of whole blood was used exclusively as the anticoagulant in collection of plasma.

The needle-plugging test was devised as a means of assessing the degree of anticoagulant effect. A No. 22 gauge, one-inch long needle with a short length of rubber tubing attached is inserted and taped in place in an antecubital vein with a blood pressure cuff inflated to 50 mm. of mercury applied proximally. As little motion of the needle as possible is achieved by holding the arm extended and motionless. Blood is allowed to drip from the end of the rubber tube into a graduated centrifuge tube which is changed at one-minute intervals in order to determine the rate of flow per minute. The procedure is continued until the flow ceases as a result of plugging of the needle with a fibrin clot or until fifteen minutes has elapsed with flow still persisting. Stained sections of the clot

which occurs in the lumen of the needle show fibrin and rare platelets. Normally, flow begins to slow in 2 to 3 minutes and ceases at 5 to 12 minutes. Patients with significant coagulation defects fail to plug the needle and flow persists for the full fifteen minutes. The test is called positive if plugging of the needle occurs in less than 15 minutes and negative if flow continues unabated at 15 minutes.

RESULTS

One-Stage Prothrombin.—Prolongation of the one-stage prothrombin time is known to reflect a deficiency of any one or more of the so-called prothrombin complex, namely, prothrombin, proconvertin, or AC globulin. Following phenindione administration the one-stage prothrombin time becomes prolonged in 12 hours and reaches a range of 26 to 36 seconds by 24 to 36 hours. Details of dose and response are outlined elsewhere.[9] The one-stage prothrombin expressed in per cent of normal usually conformed with the degree of depression of either proconvertin or prothrombin, whichever was lower.

Two-Stage Prothrombin.—In 67 cases, 44 of whom were on short-term therapy and 23 of whom were on long-term therapy, the two-stage assay was done. The one-stage prothrombin value for every case lay between 4 and 12 per cent. The results are shown graphically by days' duration of treatment in Fig. 1. It is apparent that before 8 days the two-stage value is considerably

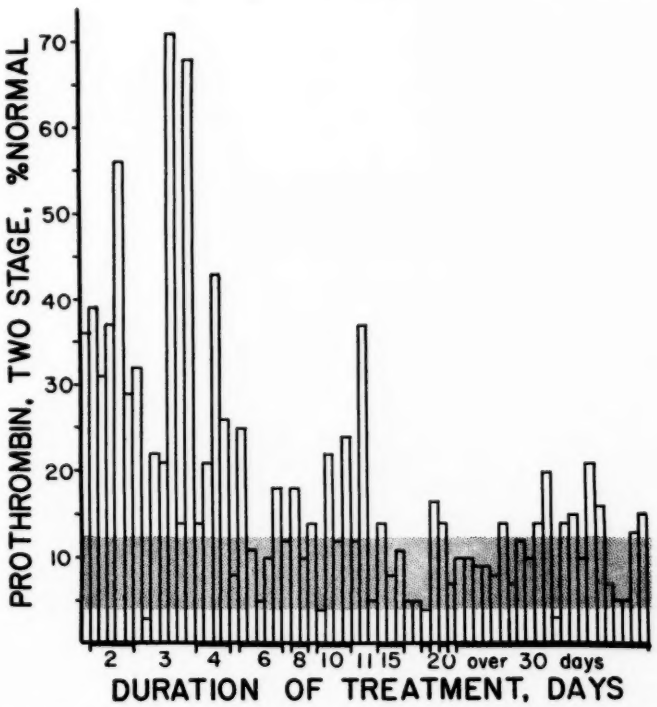

Fig. 1.—Comparison of one- and two-stage prothrombin values during treatment with phenindione. Each bar represents the two-stage prothrombin value of 67 cases, and the cases are arranged in order of duration of treatment. In all cases the one-stage prothrombin value lay between 4 and 12 per cent, which limits are represented by the shaded area. If the one-stage prothrombin test were measuring prothrombin only, the tops of the bars would fall within the shaded area. During the first week of treatment a marked discrepancy exists between these two values in the majority of cases

higher than the one-stage value in the majority of instances. In only 8 out of 24 instances did the two-stage match the one-stage value with any degree of closeness. After 8 days the agreement between the one- and two-stage values was closer. The spread of two-stage values was large compared to the one-stage values. There were still a few in the ambulatory group who showed prothrombin of over 20 per cent.

Proconvertin.—The distribution of individual values for proconvertin for all those whose one-stage value lay between 4 and 12 per cent is shown in

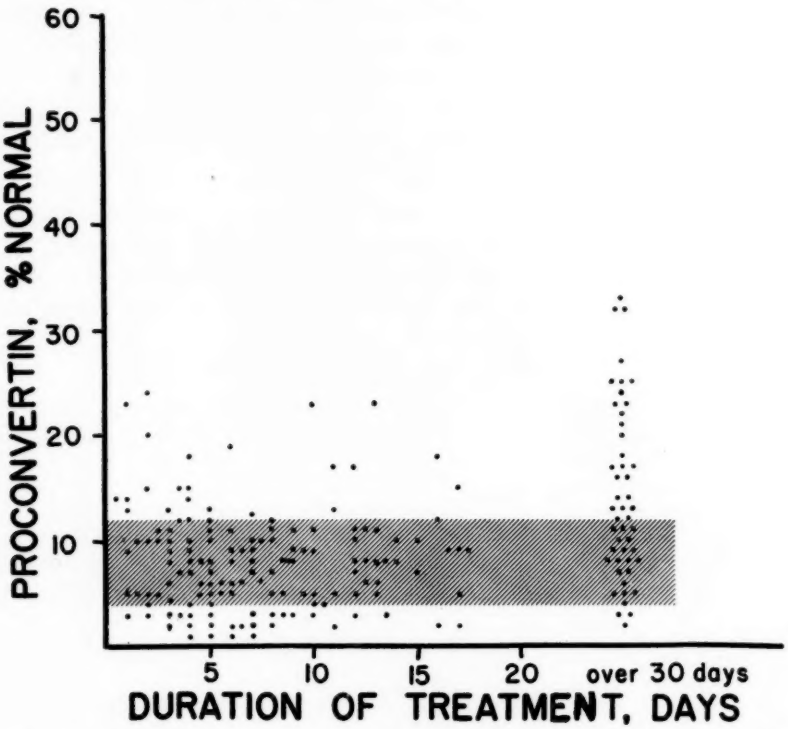

Fig. 2.—Comparison of proconvertin and one-stage prothrombin values during treatment with phenindione. In all cases the one-stage prothrombin value lay between 4 and 12 per cent, which limits are represented by the shaded area, and the cases are arranged in order of duration of treatment. There is a reasonably close agreement between the two values in the early phase of treatment in the majority of instances. In the ambulatory group (over 30 days of treatment), there is some tendency for the proconvertin values to be higher.

relation to duration of treatment in Fig. 2. Although again there are individual variations, it is readily apparent that in the majority of cases, proconvertin is depressed to a value comparable to the one-stage value immediately. There is some tendency for proconvertin to be slightly higher than the one-stage value in the long-term ambulatory group, which is represented in the chart as over 30 days' duration of treatment.

One feature of the behavior of proconvertin in relation to the one-stage prothrombin value became apparent. Those who showed a proconvertin value relatively higher than the one-stage prothrombin tended to be sensitive to

phenindione. Therefore, this observation was tested by following the proconvertin routinely along with the one-stage prothrombin in 86 patients on short-term therapy to see if this relatively simple comparison would add any important information in the management of these patients. It was found that the cases could be divided into two groups: a group called the "proconvertin pattern" where the proconvertin value and the one-stage prothrombin value agreed closely or the proconvertin value was lower than the one-stage prothrombin value during the first 3 to 4 days, and a group called the "prothrombin pattern" where the proconvertin value was distinctly higher than the one-stage prothrombin value in the first 3 to 4 days. There were 53 cases in the proconvertin pattern group, and 33 in the prothrombin pattern group.

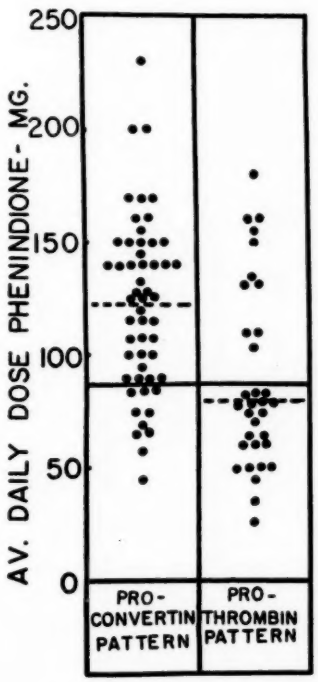

Fig. 3.—Distribution of the average daily dose of phenindione in 53 cases responding with a "proconvertin pattern" compared to 33 cases responding with a "prothrombin pattern."

The resistance to phenindione was higher in the proconvertin pattern group. For instance, the mean daily dose of phenindione for the proconvertin pattern group was 123 mg. per day and 75 mg. per day for the prothrombin pattern group. Although there was considerable overlap, 43 of the 53 in the proconvertin pattern group required more than 85 mg. of phenindione per day to maintain therapy, while only 11 of 33 of the prothrombin pattern group required this much drug. The results are illustrated in Fig. 3. In addition, those whose prothrombin time was consistently or frequently less than 24 seconds, indicating in general difficulty in producing an elevated

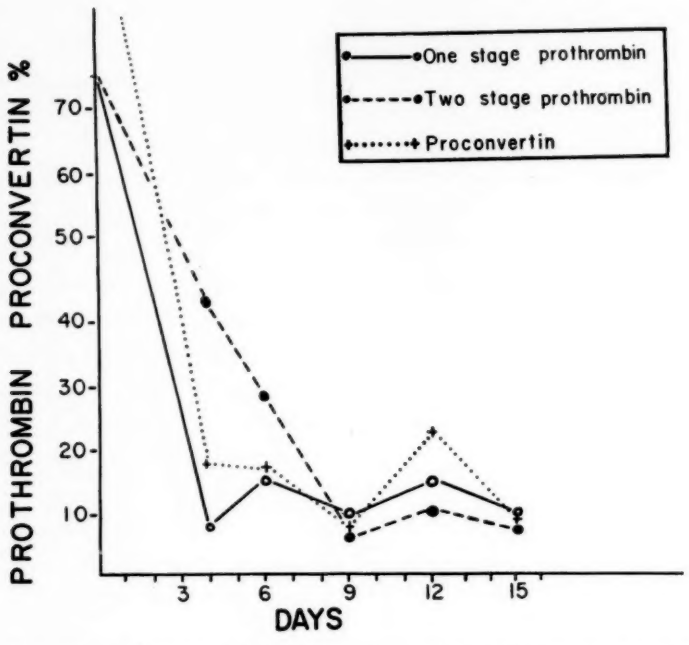

Fig. 4.—Case illustrating a predominant proconvertin deficiency in the early phase of treatment, "proconvertin pattern."

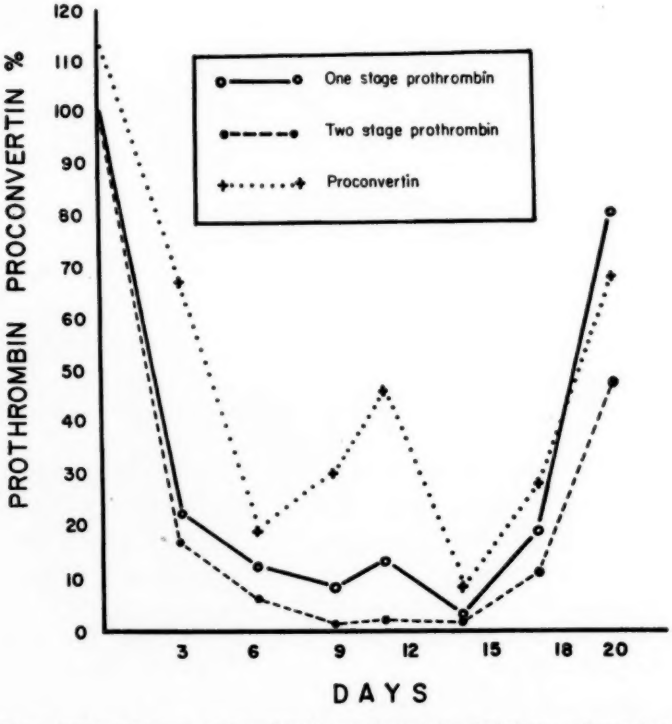

Fig. 5.—Case illustrating a predominent prothrombin deficiency throughout treatment, "prothrombin pattern."

prothrombin time because of too much caution in giving higher doses of phenindione, tended to fall almost exclusively into the proconvertin pattern group. On the contrary, those who developed excessively long prothrombin times (over 50 seconds), indicating overdosage, tended to fall into the prothrombin pattern group more often, although this difference was less striking.

When this relationship between proconvertin and one-stage prothrombin was followed beyond 3 to 4 days, it was observed that the proconvertin pattern response changed to a prothrombin pattern within 6 to 15 days. Figs. 4 and 5 illustrate cases of proconvertin and prothrombin pattern where serial determination of proconvertin, one-stage prothrombin, and two-stage prothrombin were performed. In Fig. 4 the change from proconvertin to prothrombin pattern at 9 days is shown.

Thromboplastin Complex of Plasma.—Two features shown by patients receiving phenindione led to a suspicion of a disorder of the thromboplastin complex developing after a long period of administration. These were a tendency for more easy bleeding and development of a long glass clotting time. Then the patient described below was observed.

CASE REPORT

H. S., a 66-year-old diabetic man had been treated with phenindione for over 3 years because of arterial insufficiency with impending gangrene of the foot. Minor epistaxes, hematuria, and ecchymoses were experienced when the one-stage prothrombin time was in the 26- to 36-second range. His dose therefore had been reduced so that prothrombin times ranged between 18 and 26 seconds. One day before admission to the hospital after minor injury, epistaxis began, soon followed by extensive ecchymoses, gross hematuria, tarry stools, and bleeding gums. On admission to the hospital, venipunctures and the site of a bleeding time persistently oozed in troublesome fashion. Following 100 mg. vitamin K_1 emulsion administered intravenously, bleeding ceased in 2 hours. Because of recurrence of vascular insufficiency of the foot and brain, he was started on phenindione again 3 months later without further bleeding.

Only a limited study before treatment was possible and the results are outlined in Table I. The one-stage prothrombin time and clotting time were performed immediately, but the other tests were done on oxalated plasma stored overnight. Although a deficiency existed in prothrombin and proconvertin which was greater than expected on the basis of the one-stage prothrombin, it was still not beyond the magnitude commonly seen without bleeding.

Shortening of recalcification time by normal serum compared to his own serum and failure of $BaSO_4$ plasma and PTC-deficient plasma to shorten significantly the recalcification time suggested the patient had a deficiency of PTC.

Further study of the thromboplastin complex was made on a number of patients with use of glass clotting time, prothrombin consumption, and thromboplastin generation test.

TABLE I. COAGULATION STUDIES, PATIENT H. S.

NORMAL		PATIENT
12 sec.	Prothrombin time	20.9 sec. (19%)
100%	Prothrombin two-stage method	8%
100%	Proconvertin	6%
100%	AC globulin (3 months later)	200%
7-14 min.	Glass clotting time	44 min.
–	Clot retraction	Normal
None grossly	Fibrinolysis	None grossly
3 min.	Bleeding time	∞
–	Platelets	Normal by smear
Negative	Tourniquet test (90 mm. 10 min.)	Negative
180 sec.	Plasma recalcification time	580 sec.

RECALCIFICATION TIMES

Patient's plasma 0.1 ml.; corrective factor diluted 1:10 in oxalate buffer mixture 0.1 ml.; and $CaCl_2$ 0.03 M 0.1 ml.

CORRECTIVE FACTOR	RECALCIFICATION TIME (SECONDS)
Control (oxalate buffer solution alone)	535
Normal plasma	165
$BaSO_4$ fresh normal plasma	430
Normal serum	90
Patient's serum	193
Patient's plasma 24 hr. after vit. K_1	210
Plasma of hemophilic patient	150
Plasma of PTC-deficient patient	400

Glass Clotting Time.—The results in relation to duration of treatment and one-stage prothrombin are shown in Fig. 6. This shows that the glass clotting time is only sporadically increased until administration of phenindione had been carried on for over 30 days. The clotting times of those individuals on

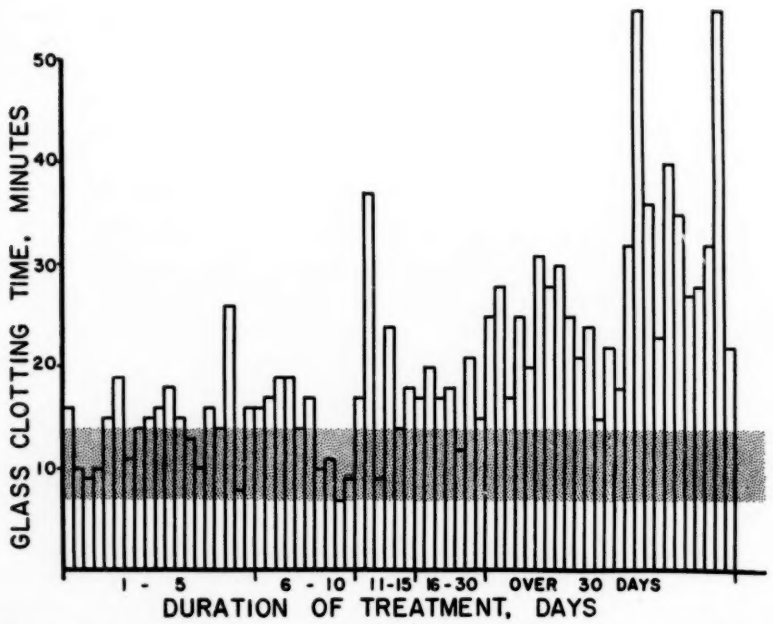

Fig. 6.—Prolongation of whole blood glass clotting time in patients receiving phenindione. The cases are arranged in order of duration of treatment and the normal values are outlined by the shaded area.

ambulatory treatment were consistently and sometimes dramatically prolonged. This was found to be so on repeated examinations. Within the limits of the values observed, there was no clear-cut relation of clotting time to one-stage prothrombin time.

Prothrombin Consumption.—Since there is a scarcity of prothrombin to consume, this test on straight whole blood was found to be not helpful. However, if the patients' blood or plasma was used as a corrective agent by adding to whole AHG- or PTC-deficient blood, the prothrombin consumption would then indicate qualitatively the presence or absence of AHG or PTC. There was normal correction of AHG-deficient blood by phenindione plasma. On the other hand 50 per cent of long-term patients on phenindione failed to correct PTC-deficient blood. The details are reported elsewhere.[8] No subjects with PTA deficiency were available for assay of this factor.

Thromboplastin Generation Test.—This was done with use of the serum of 29 patients receiving phenindione when the one-stage prothrombin activity varied from 1 to 27 per cent. The results are shown along with the one-stage prothrombin, two-stage prothrombin, proconvertin, and glass clotting time in Table II. The generation of a normal amount of plasma thromboplastin in known to depend on the presence of only a small amount of proconvertin.[15] Since there are variable amounts of proconvertin observed in this group of

TABLE II

CASE	DURATION TREATMENT (DAYS)	ONE-STAGE PROTHROMBIN (%)	TWO-STAGE PROTHROMBIN (%)	PRO-CONVERTIN (%)	THROMBO-PLASTIN GENERATION, PATIENT'S SERUM (%)	GLASS CLOTTING TIME (MIN.)
1	3	1	21	5	7	13
2	3	7	71	12.5	52	–
3	3	4	14	3	20	14
4	3	12	68	22	100	15
5	4	7	54	12	57	12
6	4	3	32	4	6	17
7	4	5	25	2	6	14
8	6	15	22	38	75	14
9	7	12	23	4.5	14	15
10	8	10	18	18	50	–
11	9	15	11.5	20	64	–
12	10	7	24	3	11	17
13	12	6	4.5	18	64	–
14	13	7	19	7	32	18
15	16	4	12	2	38	37
16	20	6	11	2	8	16
17	23	15	28	15	34	20
18	25	4	7	2	13	16
19	44	4	10	2	20	17
20	Months	12	12	11	32	25
21	Months	11	10	12	29	21
22	Months	20	18	27	32	24
23	Months	27	22	25	17	25
24	Months	12	24	12	17	22
25	Months	12	–	28	15	18
26	Months	7	–	12.5	10	32
27	Months	10	–	7.5	12	75
28	Months	19	–	29	16	36
29	Months	18	12	15	37	–

patients, it was felt that further information on the amount required to support the thromboplastin generation test could be learned. In Fig. 7, the per cent thromboplastin generated is compared with the proconvertin in two groups of patients: those who received phenindione for less than 30 days and those who received it for more than 30 days. Although 100 per cent thromboplastin is formed in only one individual, there is distinctly less formed when the proconvertin is less than 10 per cent. This relationship is seen only in patients receiving phenindione for less than 30 days. In those receiving phenindione for more than 30 days, generation of more than 34 per cent thromboplastin was not seen regardless of the height of the proconvertin within the limits of the study. This finding in the long-term cases is in accord with the previous finding of PTC deficiency developing after prolonged treatment.[8] The chronological relation of development of prolonged clotting time, evidence of PTC deficiency, and a deficiency of a serum thromboplastin factor indicate that these are all the result of a common effect. It would also indicate that the prolongation of the clotting time is a result of PTC deficiency rather than a deficiency of prothrombin or proconvertin.

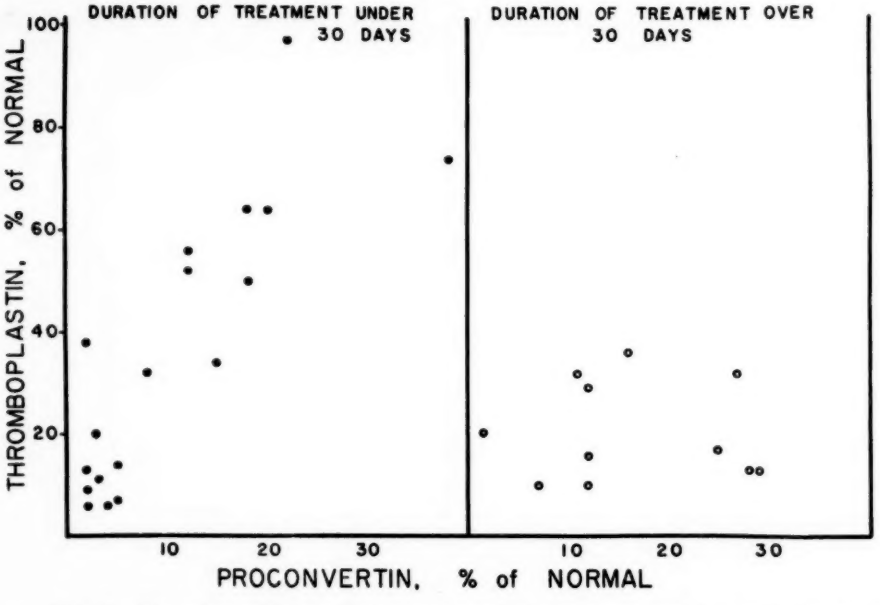

Fig. 7.—Thromboplastin generation test using the patient's serum, normal $BaSO_4$ plasma, and normal platelets. The values are compared with the proconvertin level in short- and long-term treatment. Before 30 days of treatment the value is below 20 per cent only when the proconvertin is depressed below 10 per cent. After 30 days of treatment the value is below 20 per cent in 5 of 9 cases where the proconvertin is above 10 per cent, probably as a result of PTC deficiency.

Needle-Plugging Test.—This test was done in 46 subjects and the results are listed in Table III. The test was positive in 13 of 17 patients who had received phenindione for 6 days or less. Those who had received phenindione for longer periods showed negative tests in all but 3 instances. Even in those where the one-stage prothrombin activity was greatly depressed in the early

phase of treatment, the test was positive. On the other hand, in those who had been on phenindione for more than 7 days, the test was negative, even in two instances where the one-stage prothrombin activity was as high as 20 per cent.

If the needle-plugging test is an indication of how readily a clot can form in a moving stream of blood, where the potent accelerators are constantly being washed away and where a surge of coagulation is required to overcome this washing away effect, it is apparent that no significant one exists with any frequency during the first week or so. Thereafter there are only sporadically positive tests, suggesting an adequate anticoagulant effect.

TABLE III

CASE	DURATION TREATMENT (DAYS)	ONE-STAGE PROTHROMBIN (%)	GLASS CLOTTING TIME (MIN.)	NEEDLE PLUGGING
1	1	15	14	+
2	1	12	16	–
3	2	12	10	+
4	3	3.5	9	+
5	3	3	14	+
6	3	4	19	+
7	3	6	–	+
8	3	5	11	+
9	4	8	15	+
10	4	20	13	–
11	4	3	11	+
12	4	4	14	–
13	5	4.5	12	+
14	5	8	10	+
15	5	8	12	–
16	5	9	–	+
17	6	10	13	+
18	7	6	26	–
19	7	5	8	–
20	7	12	15	–
21	9	7.5	15	–
22	9	8	16	–
23	10	4	14	–
24	11	8	10	–
25	11	8	11	–
26	11	18	7	–
27	11	4.5	24	–
28	13	7	18	–
29	13	20	9	–
30	16	4	37	–
31	17	15	9	–
32	17	6	24	–
33	19	9	14	–
34	21	18	11	+
35	Months	7	21	–
36	Months	10	15	–
37	Months	5	25	–
38	Months	24	28	–
39	Months	12	12	+
40	Months	6.5	17	–
41	Months	10	25	–
42	Months	20	20	–
43	Months	8	22	+
44	Months	7	31	–
45	Months	10	28	–
46	Months	4	67	–

DISCUSSION

Heparin is considered to have a true anticoagulant effect because heparinized blood can stand in glass tubes for a long period of time or pass through tubing such as that of an artificial kidney or extracorporeal pump without clotting. No such effects are as dramatically apparent with the prothrombopenic anticoagulants. Their clinical use has been based on the assumption that prolongation of the one-stage prothrombin time to arbitrary values will make coagulation more difficult. The fact that bleeding occurs under certain circumstances, however, must mean that there is a definite interference with the coagulation mechanism, and clinical results indicate that an anticoagulant effect can be achieved. In light of the present findings, certain general conclusions can be made regarding the effect of phenindione on the coagulation mechanism. First, it is readily apparent that the one-stage prothrombin test does not reflect the changing pattern of the coagulation mechanism that takes place with the passage of time. In one instance there is only a proconvertin deficiency and no anticoagulant effect while in another with an identical one-stage prothrombin value there are a variety of deficiencies, long glass clotting time, and adequate evidence of anticoagulant effect. There is a wide scatter of values individually, but in general the effect of phenindione can be described by 3 chronological phases which are outlined in Table IV.

TABLE IV. USUAL PATTERN OF THE COAGULATION MECHANISM AS EFFECTED BY PHENINDIONE

DURATION OF TREATMENT	PHASE I (1-7 DAYS)	PHASE II (8-30 DAYS)	PHASE III (OVER 30 DAYS)
Two-stage prothrombin	High	Low	Low
Proconvertin	Low	Low	Slightly higher
Glass clotting time	Normal	Normal	Prolonged
Needle-plugging test	Positive	Negative	Negative
Plasma thromboplastin component	Normal	Intermediate	Low

It appears as a result of infusion experiments that both prothrombin[4] and proconvertin[16] have rapid turnovers in vivo. Thus if their production was stopped, a deficiency would develop rapidly. On the other hand, infusion experiences with PTC suggest that this factor has a long turnover time.[17, 18] In our experience the effect of an infusion of plasma in a PTC-deficient patient may be felt in prothrombin consumption, and the thromboplastin generation tests as long as two weeks later. Therefore if production of PTC was stopped, a deficiency would develop slowly. Since all degradation curves are asymptotic, an intermediate deficiency of PTC should be present by 10 days, but since this would not be measurable until the value falls below somewhere in the neighborhood of 20 to 25 per cent, it would not be detected until late in the course of treatment when such levels are likely to exist since it is present in only half of the subjects.[8] In regard to inhibition of another thromboplastic factor, as suggested by Walker and Hunter[6] and Fluckiger and co-workers,[7] there is nothing in our studies to either support or disprove it.

Speculation might be made as to which individual deficiency is responsible for the anticoagulant effect. Proconvertin deficiency as seen in Phase I does

not appear to be associated with a measurable anticoagulant effect. This is logical on the basis of current concepts of blood coagulation. According to Owren[19] only a small amount of proconvertin is used in spontaneous clotting of blood in glass tubes, 90 per cent or more of the plasma proconvertin remaining unused in the serum after coagulation. When more thromboplastin is added, then more proconvertin is converted to convertin, until finally when there is added an excess of thromboplastin (such as in the one-stage prothrombin test) presumably all of the proconvertin may be changed. This suggests that plasma in the process of spontaneous clotting forms a relatively weak thromboplastin* compared to that used in the one-stage prothrombin test; and if the relationship between the two factors is stoichiometric, a very extreme deficiency of proconvertin is necessary before spontaneous coagulation would be inhibited. This point is evident in cases of congenital deficiency of proconvertin[16, 20-23] where prothrombin times of 55 to 70 seconds are encountered with normal whole blood clotting times. We have observed one patient with a one-stage prothrombin time of 40 seconds who had no bleeding tendency. This is further evidence that proconvertin is present in large excesses. It is difficult to interpret nature's reasons for this excess, as well as the even greater excess that occurs during pregnancy.[24]

Simultaneously with the appearance of prothrombin deficiency in Phase II there develops an anticoagulant effect as measured by the needle-plugging test. This may be due to prothrombin deficiency alone, a combination of prothrombin and proconvertin deficiency, the early partial and undetectable development of PTC deficiency, or a combination. Although it is impossible to be sure which of these factors individually is responsible for the anticoagulant effect, the occasional negative needle-plugging tests in the presence of fairly high values of two-stage prothrombin and the aforementioned reasons for believing that the proconvertin deficiency alone is an unlikely cause suggest an intermediate PTC deficiency or a combination of deficiencies as the basis for the anticoagulant effect. On the other hand, the correlation of change in needle-plugging test from positive to negative at the same time the prothrombin is seen to become depressed points to a deficiency of this factor as an important one in development of an anticoagulant effect.

Olwin[25] recommended the use of the two-stage prothrombin in the control of Dicumarol therapy and the basis of this becomes more reasonable with these findings in mind. Further study with more quantitative methods of measurement of PTC are necessary to further elucidate the problems.

The question of whether PTC deficiency is a desirable or undesirable complication of treatment is raised. The hemorrhagic pattern of those on long-term anticoagulants resembles PTC deficiency in some respects—namely, easy bruising, occasional hematomas, and the rare hemarthrosis. On the other hand, epistaxis, hematuria, and positive tourniquet test are features characteristic of the patient on the anticoagulant. If the anticoagulant effect is adequate

*"Thromboplastin" is used in the sense of the factor which reacts with proconvertin and calcium to form convertin and not in the sense that Biggs and Douglas use, namely, the factor which converts prothrombin to thrombin.

from prothrombin deficiency alone, and if PTC deficiency is a significant factor in development of bleeding, then the PTC depression is an undesirable side effect. If, on the other hand, the PTC depression is an essential part of the anticoagulant effect, it is desirable. Again further investigation of this relationship is needed.

Certain recommendations regarding the use of anticoagulants can be made. First, these studies further support the concept that phenindione (and probably the same holds true for Dicumarol) is ideally suitable for long-term anticoagulation where there is a distinct and broad effect on the coagulation mechanism. Second, whenever an immediate anticoagulant effect is desired, heparin should be used. Also if there is to be a shift from one to the other it would be sound to overlap heparin and phenindione by at least 6 days. Third, the use of the proconvertin related to the one-stage prothrombin may be of some use in predicting dose requirements. This in effect, however, has not been helpful because the information gained precedes the same information from the one-stage procedure alone by only one or two days.

SUMMARY

The one- and two-stage prothrombin, proconvertin, glass clotting time, thromboplastin generation test, and needle-plugging tests were studied in a group of hospitalized patients on short-term anticoagulant therapy and in ambulatory patients on long-term therapy with use of phenindione for the prevention of spontaneous intravascular clotting.

The results indicate three chronological phases of effect on the coagulation mechanism which were apparent from group analysis, although considerable individual variation existed. Phase I lasted for about 8 days and was typified by high prothrombin, low proconvertin, normal glass clotting time, and no anticoagulant effect as judged by the needle-plugging test. Phase II lasted from 8 to 30 days and was associated with low prothrombin, low proconvertin, sporadically elevated glass clotting time, and the appearance of a significant anticoagulant effect. Phase III after 30 days or more of treatment was characterized by low prothrombin, low proconvertin, long glass clotting time, low PTC, and positive anticoagulant effect.

Patients sensitive to phenindione tend to show a one-stage prothrombin activity which is less than the proconvertin during the first few days of treatment.

Proconvertin has to be reduced to less than 10 per cent before there is significant interference in the thromboplastin generation test.

Inhibition of proconvertin alone to levels usually seen in Dicumarol and phenindione therapy has no measurable anticoagulant effect by the needle-plugging test.

It is postulated that phenindione acts by inhibiting production of proconvertin, prothrombin, and plasma thromboplastin component. Due to different survival rates of these proteins, deficiencies develop at different rates. The complete effect of phenindione is not seen for over one month.

On the basis of the findings, phenindione and similar preparations are considered particularly suited for long-term use. In short-term use heparin should be given for the immediate effect if desired and overlapped by 7 days.

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THE GLUTATHIONE INSTABILITY OF DRUG-SENSITIVE RED CELLS

A New Method for the In Vitro Detection of Drug Sensitivity

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WHEN primaquine and certain other drugs such as sulfanilamide, acetanilid, or promizole are administered to some individuals, an acute hemolytic anemia develops.[1] Earlier studies demonstrated that this type of drug sensitivity is due to an intrinsic defect of the red blood cell, a defect that is rare among Caucasians but occurs in about 10 per cent of American Negroes.[2] Drug sensitive erythrocytes appear to be perfectly normal when examined by standard hematologic techniques: (1) the morphology of the cells is normal; (2) they do not sickle, nor do they contain any abnormal hemoglobin detectable by paper electrophoresis at pH 8.6 or by alkali denaturation; (3) there is nothing characteristic about their antigenic pattern and the Coombs test is negative; (4) they are not unusually susceptible to acid hemolysis; (5) the amount of methemoglobin is normal; and (6) their mechanical, chemical, and osmotic fragility is normal.[3]

We have, however, detected two in vitro differences between sensitive and nonsensitive cells, in the course of our studies. The first of these differences is in the pattern of Heinz body formation when the cells are incubated with acetylphenylhydrazine and certain other compounds under standardized conditions. Many small scattered Heinz bodies are formed in sensitive red cells; only a few, slightly larger, marginal, Heinz bodies are formed in nonsensitive cells. When this in vitro test for drug sensitivity was applied to the blood of 104 healthy volunteers, only two false positive and no false negative tests were obtained. The test is very simple to perform. It was found, however, that the test results were quite sensitive to alterations in the degree of oxygenation of the red cells, the test being standardized at the oxygen saturation of venous blood, and that the test results also depended to a certain extent on the hematocrit.[4] Furthermore, a certain degree of subjectivity was involved in assessing the test results. It was therefore not too surprising that under field conditions this test proved somewhat less satisfactory than it had when originally carried out under carefully controlled conditions in healthy volunteers.[5]

The other difference between sensitive and nonsensitive red cells is that the average reduced glutathione (GSH) level is lower in sensitive than in nonsensitive red cells.[6] This fact has been of great interest to us but has never

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seemed to constitute a valid basis for in vitro differentiation between sensitive and nonsensitive individuals: the difference between the GSH content in sensitive and nonsensitive red cells is relatively small and there is some overlap. Furthermore, it is well known that various disease conditions may either elevate[7] or depress[8] the red cell GSH content.

Recent studies have shown a striking difference between the in vivo behavior of sensitive and nonsensitive red cell GSH. When primaquine was administered to nonsensitive subjects, there was no change in the red cell GSH level. When primaquine was administered to a sensitive subject, however, there was an abrupt fall in the GSH content of the red blood cells to about one-half of the original, already subnormal value. This abrupt fall preceded the major portion of the hemolytic reaction, and as hemolysis progressed the GSH again rose to the original level.[9]

In the current study we have succeeded in reproducing this phenomenon in vitro. In so doing, we have developed a test for red cell sensitivity which, although technically somewhat more laborious than the previously described test employing Heinz body formation, appears to be more reliable. We have also laid the basis for further in vitro study of the biochemical course of events leading to the destruction of sensitive red cells during drug administration.

MATERIALS AND METHODS

A. *Test Subjects.*—

Group 1:* Blood samples were obtained from inmates or former inmates of Stateville penitentiary. The response of these volunteers to primaquine administration had been determined previously by the actual administration of 30 mg. primaquine daily for at least seven days. None of these subjects had received drug for several weeks; the majority had not received drug for a period of from several months' to several years' duration. Of the 5 sensitive donors all were Negroes; of the 7 nonsensitive donors 5 were Negro and 2 were Caucasian.

Group 2: Blood samples were obtained from 19 Negro and 31 Caucasian adults from among the employees, outpatients, and inpatients of the University of Chicago Clinics, and from 106 Negro outpatients from the Mercy Hospital Out-Patient Clinic.† This group of subjects therefore includes both normal individuals and those with a variety of hematologic and nonhematologic disorders. Among the Negro subjects tested many were very light-skinned so that there was undoubtedly a considerable degree of racial mixture.

B. *Preparation of Tubes Containing Acetylphenylhydrazine, Phenylhydrazine, Primaquine, and Ascorbic Acid.*—Sufficient absolute methyl alcohol is added to a weighed sample of acetylphenylhydrazine to make a solution containing 100 mg. per milliliter. Appropriate quantities of this solution are pipetted to the bottom of 15 × 125 mm. test tubes, which are then dried in an 80 to 90° C. drying oven. They were stored in darkness until use. No deterioration in the activity of the acetylphenylhydrazine was observed over a period of at least three weeks. Tubes containing phenylhydrazine hydrochloride, primaquine diphosphate, and ascorbic acid were prepared by dispensing weighed quantities of these compounds directly into 15 × 125 mm. tubes.

C. *Reduced Glutathione (GSH) Determinations.*—Grunert and Phillips[10] have described a method of GSH determination which depends on the development of a rose-colored complex

*Most of these samples were obtained through the courtesy of Dr. C. Larkin Flanagan and Dr. Alf S. Alving.

†Obtained through the courtesy of Dr. Raymond J. Dern.

with sodium nitroprusside and its stabilization with cyanide. We have found the following modification most satisfactory for our purposes:

> Two milliliters of distilled water is added to 1 ml. of whole blood.* After a few minutes 5 ml. of a 3.0 per cent metaphosphoric acid solution† is added during constant agitation of the hemolysate with a stirring rod. Approximately 3 Gm. of sodium chloride is added and the test tube is sealed with Parafilm‡ and is shaken for 10 minutes in a Burrell wrist-action shaker, Model BB, set at the maximum amplitude. The suspension is filtered through paper (Whatman No. 2 and S & S No. 595 have both been satisfactory). These manipulations are all carried out at room temperature. Two milliliters of the filtrate is added to a 25 mm. Coleman Junior spectrophotometer cuvette containing 6 ml. of saturated sodium chloride solution. The mixture is allowed to equilibrate for 10 to 20 minutes at 21 to 23° C. in a water bath. A 2 per cent solution of sodium nitroprusside (prepared daily in a dark bottle) and a solution containing 328 mg. per cent (0.067 M) sodium cyanide and 15.9 Gm. per cent (1.5 M) sodium carbonate are also placed in the water bath. One milliliter of the sodium nitroprusside solution is layered over the filtrate-saline mixture. One milliliter of the sodium cyanide-sodium carbonate solution is added immediately, and the contents of the cuvette are mixed simultaneously with a stirring rod. Within one minute, the optical density at 520 μ is measured in a Coleman Junior spectrophotometer against a reagent blank containing 6 ml. of saturated sodium chloride solution, 2 ml. of a 2.0 per cent metaphosphoric acid in saturated sodium chloride solution, 1 ml. of the 2.0 per cent sodium nitroprusside, and 1 ml. of the sodium cyanide-sodium carbonate mixture. This blank is stable for at least one hour. A GSH standard equivalent to the filtrate from blood containing 50 mg. per cent GSH is prepared by dissolving sufficient reduced glutathione in 1.875 per cent metaphosphoric acid to give a concentration of 6.25 mg. per cent, saturating this solution with sodium chloride, and filtering. The standard is stored at −5° C. It is stable for several weeks and is checked periodically against a freshly prepared standard solution. The addition of 0.01 per cent disodium ethylene diaminetetraacetate to the standard appears to enhance the stability of the glutathione, presumably by chelating traces of metallic ions which might otherwise catalyze the auto-oxidation of the GSH. One or more 2 ml. aliquots of the standard are included with blood filtrates prepared each day. The GSH content is calculated according to the linear relationship that exists between optical density of the GSH-nitroprusside complex and GSH concentration. The GSH content of the red cells equals the whole blood GSH divided by the hematocrit of the sample, since almost all of the blood GSH is in the red blood cells.

D. *The "GSH Stability Test."*—The procedure described below was used to test the stability of red cell GSH to acetylphenylhydrazine and the other compounds used. This basic method was used in the experiments reported here, except where modifications are specified. It will be referred to as the "GSH stability test."

> Heparinized venous blood samples are used within 3 hours of the time they are drawn. The blood is mixed and oxygenated by shaking vigorously in 24 mm. screw-cap vials. After the red cell glutathione level has been determined in the usual manner, 1 ml. aliquots of whole blood are added to test tubes containing

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*In the original method described by Grunert and Phillips,[10] and in our earlier modification[6] a saponin solution was used. A batch of saponin which we have recently had occasion to use caused a marked decrease in GSH, some of the loss of activity being immediate and some progressive deterioration occurring with standing. We have found that mixing 1 ml. of whole blood with 2 ml. of distilled water gives results equivalent to those obtained by more completely lysing the red cells by freezing and thawing. Both lysis with distilled water and lysis by freezing and thawing gave significantly higher GSH values than saponin lysis.

†Three grams Reagent Acid Phosphoric Meta (Merck) (35 per cent HPO_3) per 100 ml. solution.

‡Marathon Corporation.

5 mg. acetylphenylhydrazine. A glass stirring rod is used to aid in the suspension of the acetylphenylhydrazine, and the tube is agitated by vigorously tapping its bottom against the palm of the hand to assure solution of the acetylphenylhydrazine and adequate oxygenation of the red cells. The tube is incubated at 37° C. The agitation is repeated at the end of one hour, and at the end of two hours GSH determinations are made in the usual manner. Precipitation of the protein with metaphosphoric acid and salt is carried out in the same tubes in which the blood has been incubated, because preliminary experiments had shown that the presence of acetylphenylhydrazine did not interfere with determination of GSH by the method employed.

EXPERIMENT AND RESULTS

A. *The Effect of Time and of Concentration of Acetylphenylhydrazine on GSH of Sensitive and Nonsensitive Erythrocytes* (Fig. 1).—A GSH determination was carried out on the blood from a known sensitive (Group 1) donor. One milliliter aliquots were pipetted into test tubes containing 1, 5, 10, or 20 mg. of acetylphenylhydrazine and into tubes containing no acetylphenylhydrazine. These were incubated at 37° C. for one-half to 4 hours, agitation being carried out at hourly intervals. GSH determinations were performed on tubes containing 5, 10, and 20 mg. of acetylphenylhydrazine after one-half hour. GSH determinations were performed on all tubes after an incubation time of 2 hours and 4 hours.

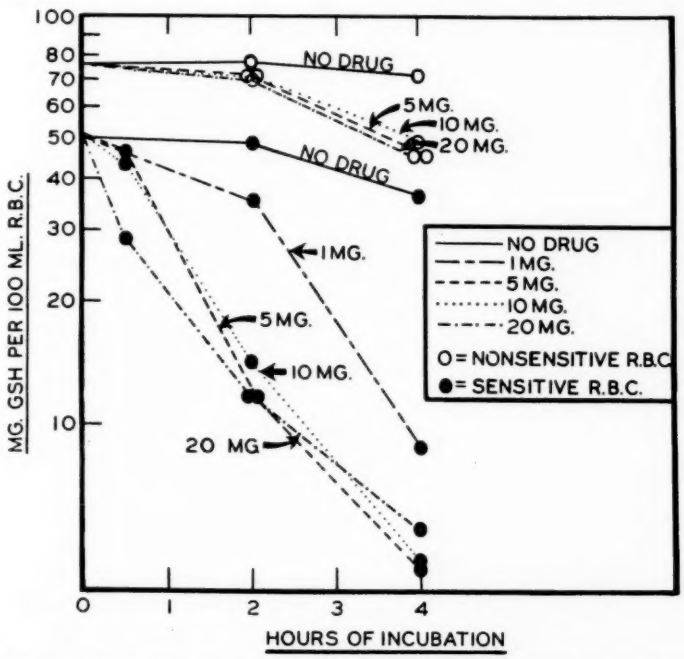

Fig. 1.—The effect of incubation with various concentrations of acetylphenylhydrazine on the GSH of sensitive and nonsensitive red cells.

A GSH determination was also carried out on blood from a normal Caucasian (Group 2). One milliliter aliquots were pipetted into tubes containing 5, 10, or 20 mg. of acetylphenylhydrazine and into tubes containing no acetylphenylhydrazine. GSH determinations were carried out on some tubes with each

concentration of acetylphenylhydrazine used after two and after four hours of incubation at 37° C. All tubes were agitated at hourly intervals.

The GSH content of sensitive red cells fell moderately when they were incubated with 1 mg. acetylphenylhydrazine per milliliter of whole blood. With 5, 10, and 20 mg. acetylphenylhydrazine, the fall in GSH level was very marked. In contrast, the GSH content of nonsensitive cells was virtually unaffected by incubation with as much as 20 mg. acetylphenylhydrazine for 2 hours, and even at the end of 4 hours the decrease in GSH content was slight.

B. *The Stability of Red Cell GSH to Acetylphenylhydrazine as a Test for Drug Sensitivity.*—The results of application of the "GSH stability test" to blood samples from 5 known sensitive and 7 known nonsensitive individuals are presented in Fig. 2 and in Table I. It is apparent that a marked fall in GSH level took place in the red cells of all of the sensitive, but not the nonsensitive individuals tested. It is of interest that both individuals reported to give false positive tests with the Heinz body procedure described earlier[4] are included in this group.

The results of the application of the GSH stability test to blood samples from 159 subjects whose response to drug administration was unknown are presented in Fig. 3 and Table I. It is clear that incubation of these samples with 5 mg. acetylphenylhydrazine distinctly divided them into two groups. One of these groups is composed of the 6 blood samples in which the GSH concentration dropped abruptly. In the other group, consisting of the remaining 153 samples, the GSH content changed very little. It is clear that this division into two groups is very similar to that observed when the test was carried out on known nonsensitive and known sensitive individuals. The difference between the initial GSH level of known sensitive cells and that of the group of 6 which showed a marked drop in the GSH level is not explained. However, it is notable in this regard that the initial GSH level of this group does correspond very closely to the value of 45.6 mg. per cent that we have previously reported as being the average red cell GSH level of 8 known sensitive individuals.[6]

TABLE I. THE RESULTS OF THE GSH STABILITY TEST ON 171 SUBJECTS

	GROUP	NUMBER	RACE	MG. % GSH PER 100 ML. RED CELLS BEFORE INCUBATION			MG. % GSH PER 100 ML. RED CELLS AFTER INCUBATION WITH 5 MG. ACETYLPHENYLHYDRAZINE FOR 2 HOURS		
				AVERAGE	SAMPLE STANDARD DEVIATION	RANGE	AVERAGE	SAMPLE STANDARD DEVIATION	RANGE
1*	Nonsensitive	7	5 Negro 2 Caucasian	66.3	9.6	48.2-81.5	60.3	10.5	44.9-81.5
1*	Sensitive	5	5 Negro	53.4	6.9	47.6-63.6	9.4	2.4	5.9-11.9
2*	Little change in GSH	153	121 Negro 32 Caucasian	67.3	14.7	43.1-151.1	64.9	14.4	35.9-137.0
2*	Marked drop in GSH	6	6 Negro	42.8	4.0	35.2-44.4	9.1	4.2	4.8-15.2

*See Materials and Methods.

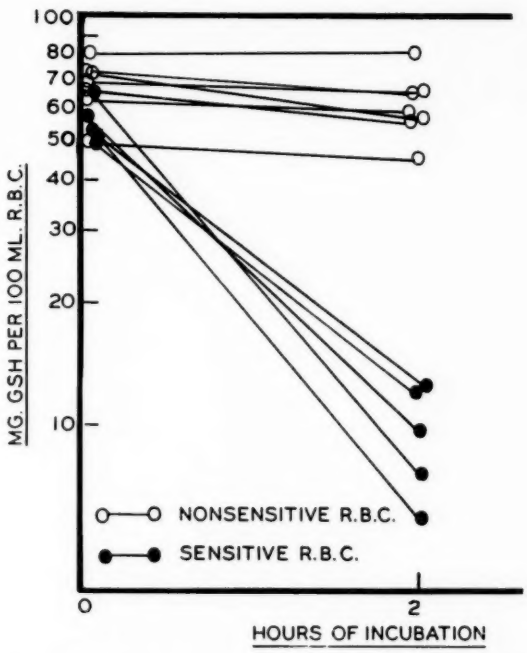

Fig. 2.—The results of the "GSH stability test" on blood samples from 7 known nonsensitive and 5 known sensitive individuals.

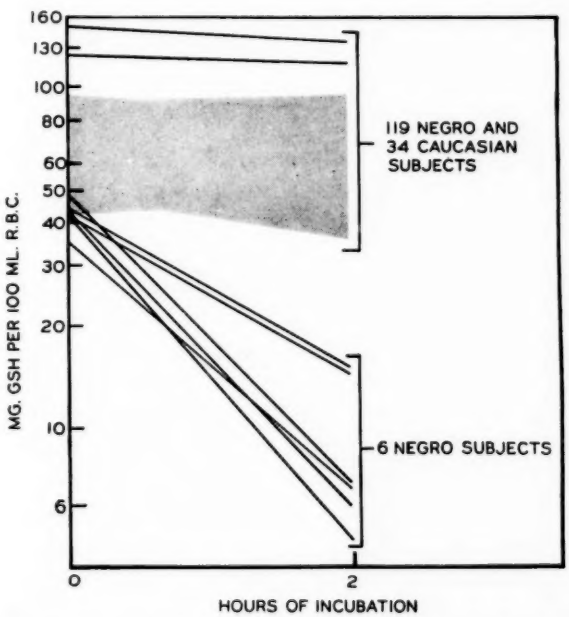

Fig. 3.—The results of the "GSH stability test" on blood samples from 159 subjects; drug-sensitive status unknown.

The pooled data from all 171 subjects, including those of known and of unknown sensitivity to primaquine, demonstrate that after incubation with acetylphenylhydrazine the GSH concentration falls into either one of two groups (Fig. 4). No intermediate values were observed.

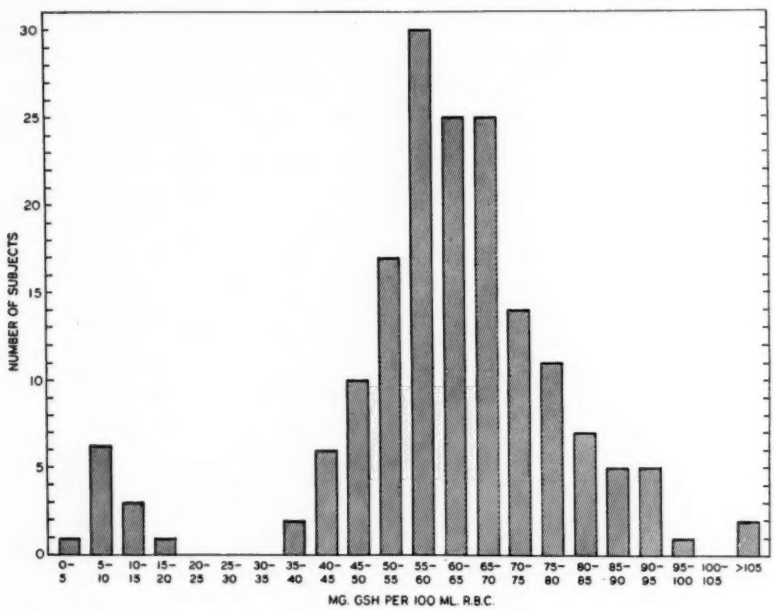

Fig. 4.—The GSH content of the red blood cells from 171 subjects after incubation with acetylphenylhydrazine for 2 hours.

C. *The Effect of the Degree of Oxygenation on the Stability of GSH of Sensitive and Nonsensitive Red Cells.*—A sample of blood from a known sensitive individual and a sample of blood from a normal Caucasian were each divided into three parts. One aliquot of each was shaken with a large volume of air, one with carbon dioxide, and one with carbon monoxide. GSH stability tests were carried out in the usual manner, the space above the incubating blood being flooded with the gas with which it had originally been shaken. The GSH of nonsensitive cells was stable in each of the three preparations, slightly more so in the presence of CO and CO_2 than with air. The red cells shaken with air gave slightly lower GSH levels than did the blood mixed with CO_2 and considerably lower than blood mixed with CO.* The results of this experiment are presented in Fig. 5. The effect of oxygen on the GSH level of sensitive cells incubated with acetylphenylhydrazine was much more striking (Fig. 6). The blood sample that had been shaken with air showed the characteristic fall in the GSH level that was observed whenever blood from a known sensitive individual was subjected to the "GSH stability test." The fall in the GSH level with CO_2 was much less, and there was no fall in the aliquot that had been mixed with carbon monoxide.

*Presumably, a certain amount of auto-oxidation of glutathione takes place in the preparation of the filtrate and this loss of GSH may be prevented by displacing the oxygen.[11]

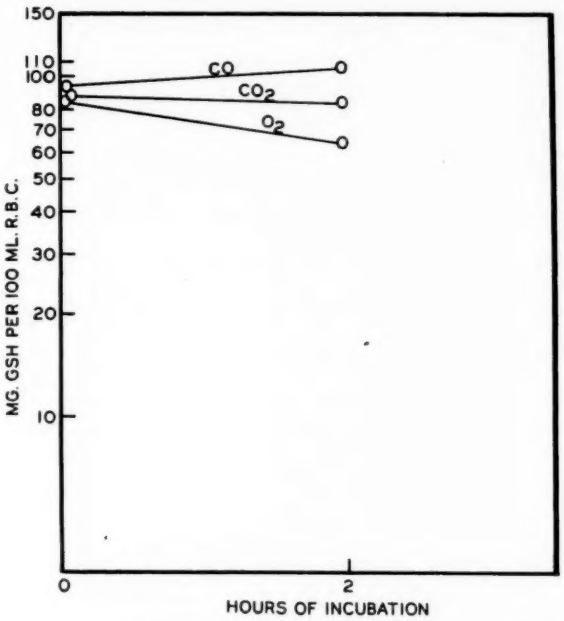

Fig. 5.—The effect of O_2, CO_2, and CO on the "GSH stability test" applied to sensitive red cells.

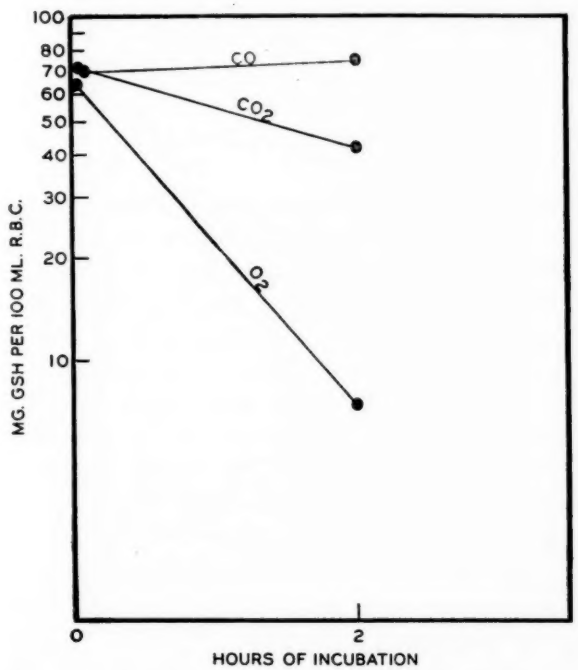

Fig. 6.—The effect of O_2, CO_2, and CO on the "GSH stability test" applied to nonsensitive red cells.

D. *The Effect of Hematocrit.*—The hematocrit of the blood of a sensitive and of a nonsensitive donor were altered by separating the plasma and cells by centrifugation and mixing varying quantities of packed cells and plasma. The hematocrit of each of these mixtures was measured, and the GSH stability test was carried out. It was apparent that over the wide range studied, hematocrit had no influence on the results of the test.

TABLE II. THE EFFECT OF STORAGE ON THE CAPACITY OF NONSENSITIVE RED CELLS TO PROTECT GSH

STORAGE TEMPERATURE (C.)	TIME (HRS.) STORED BEFORE INITIATION OF GSH STABILITY TEST	GSH STABILITY TEST	
		INITIAL GSH (MG./100 ML. R.B.C.)	GSH AFTER 2-HR. INCUBATION WITH ACETYLPHENYLHYDRAZINE (MG./100 ML. R.B.C.)
–	0	57.5	59.3
4°	2	59.9	63.7
	4	58.4	66.2
	6	64.3	64.9
	24	62.5	31.9
22°	2	64.9	66.2
	4	59.6	67.1
	6	61.7	52.6
	24	55.6	5.2
	24 with added glucose	56.4	55.8
37°	2	62.8	62.3
	4	61.3	17.3
	6	62.8	10.7
	24	20.5	1.7

E. *The Effect of the Storage Time and Temperature of the Blood Sample.*—Blood from a normal Caucasian was divided into four parts. One of these was stored at 37° C., one at 22° C., and one at 4° C. To the fourth sample sufficient glucose was added to increase the final glucose concentrations by 400 mg. per cent. It was stored at 22° C. GSH stability tests were carried out initially and on each of the blood samples without added glucose after 2 hours, 4 hours, 6 hours, and 24 hours of storage. After 24 hours at 22° C., a GSH stability test was also performed on the portion to which glucose had been added. The results of this experiment are given in Table II. They indicate that the capacity of normal red cells to protect glutathione was lost after 4 hours of incubation at 37° C., to a slight extent after 6 hours at 22° C., and to a much greater extent after 24 hours at 22° C., but only slightly after 24 hours at 4° C. A glucose supplement prevented loss of the capacity of these cells to protect their glutathione content after 24 hours of incubation at 22° C.

F. *The Stability of GSH of Sensitive and Nonsensitive Cells to Other Compounds.*—The stability of sensitive and nonsensitive red cell GSH in 1 ml. of blood to 2 and 5 mg. of phenylhydrazine hydrochloride, 1 and 2 mg. ascorbic acid, 6 and 10 mg. of primaquine diphosphate, 0.01 ml. of aniline, and 1 mg. of hydroxylamine was also tested. In each case, a larger proportion of the glutathione of sensitive cells was destroyed than was the case with nonsensitive

cells under identical conditions. It was noted that with several of the compounds tested, the higher concentration tested caused less glutathione destruction, both in sensitive and in nonsensitive cells, than did the lower concentration. Although the reason for this finding is not yet clear, it may be due to reduction of oxidized glutathione by an excess of the reducing compound used. An incidental observation was that incubation with primaquine diphosphate caused clotting of heparinized blood.

DISCUSSION

A clear-cut difference between the in vitro stability of the GSH of red cells from 5 known sensitive and 7 known nonsensitive individuals to incubation with acetylphenylhydrazine has been demonstrated. Although the number of such individuals from whom blood samples have been tested is small, the differentiation is so clear that there is little doubt of the validity of the observation. Application of this technique, under field conditions, to blood samples from 159 other individuals has resulted in clear-cut differentiation into two groups. The GSH stability of the red cells of one of these groups closely approximated that of the known sensitive individuals, the other of the known nonsensitive individuals. The former group consisted of 4.8 per cent of the Negroes tested. Previously, we found 22 sensitive individuals among 199 consecutive American Negroes given primaquine, approximately 11 per cent.[12] These figures differ significantly from each other, but the difference may readily be explained on the basis of the marked degree of racial mixture of our present "Negro" subjects. Taking this factor into consideration, the agreement between these figures seems reasonably good. None of the 31 Caucasians tested showed the abrupt fall of GSH that was characteristic of the known sensitive subjects. This agrees well with the extremely low incidence of sensitivity among Caucasians (probably less than 1 or 2 per 1,000).[12]

Although somewhat more laborious, the GSH stability test described in this paper appears more satisfactory in several respects than our previously described technique[4] of in vitro differentiation between sensitive and nonsensitive cells: (1) it is standardized at almost complete oxygenation, a more reproducible condition than the partial oxygenation at which the earlier technique was standardized; (2) it is virtually independent of hematocrit (except in the calculation of the red cell GSH content); (3) the interpretation of results is entirely objective; (4) the spread between positive and negative results is much wider; and (5) the two individuals who gave unexplained false positive results with the earlier test clearly fall into the nonsensitive group with the new technique.

It is apparent from the earlier evidence already cited and from the data obtained in this study that there is a close relationship between red cell GSH and sensitivity to hemolysis.

The exact nature of this relationship between GSH and sensitivity to hemolysis has not yet been clarified. When we first found that the level of glutathione in sensitive cells was only about two-thirds of that in nonsensitive cells, it appeared possible that the fundamental defect of sensitive cells might merely

be a deficiency in glutathione, and that in the absence of its protective properties, the cell became unusually susceptible to the denaturing effect of the hemolytic drugs. The effect of the sulfhydryl-blocking agents, iodoacetate, and arsenite, in enhancing Heinz body formation in nonsensitive cells incubated with acetylphenylhydrazine appeared to support this theory. In the light of subsequent studies, however, this view no longer seems tenable. Reduced glutathione determinations have been carried out on samples of blood from sensitive individuals who had undergone drug-induced hemolysis and were temporarily completely resistant to the hemolytic effect of the drug. Although these individuals were nonsensitive to hemolysis,[13] the GSH content of their red cells was still abnormally low.[9] If diminished red cell GSH content were the sole cause of drug sensitivity, these individuals should have been sensitive. Furthermore, the simple concept that GSH concentration alone governs sensitivity to hemolysis fails to explain the difference between the stability of the glutathione of sensitive cells to drugs both in vivo and in vitro. If GSH does serve in a protective capacity, one must also postulate that nonsensitive cells have a mechanism which protects GSH and that in sensitive cells this mechanism is defective. Although it could be argued that the falling GSH level in the red cell merely serves as a convenient indicator of the destructive changes that are taking place, this seems quite unlikely. The GSH level of sensitive cells is abnormally low before drug has ever been administered and the fall in GSH level precedes any other detectable change during drug administration to a sensitive subject.

In addition to its utility in the detection of drug sensitivity, the technique described in this paper forms an excellent basis for the in vitro study of this first stage of the hemolytic process, the fall in GSH concentration. We are, therefore, carrying out further studies of its mechanism, the nature of the reaction that leads to the destruction of GSH, its end products, and the biochemical processes that protect GSH in nonsensitive cells. These studies will be reported in a subsequent communication.

SUMMARY

1. A "GSH (reduced glutathione) stability test" has been described. This test consists of incubating blood samples with acetylphenylhydrazine and measuring the GSH level before and after incubation.

2. Application of this test to the blood of five known drug-sensitive and seven known drug-nonsensitive individuals demonstrated that the glutathione of sensitive cells is unstable, while that of nonsensitive cells is stable.

3. Application of the test to 159 subjects, drug sensitive status unknown, demonstrated that these individuals were divided clearly into two groups. The incidence and radial distribution of these groups approximated the known incidence and racial distribution of drug sensitivity.

4. The GSH instability of sensitive red cells depends upon the presence of oxygen.

5. The results of the test are independent of hematocrit over a wide range.

6. Glucose is required for the protection of GSH by nonsensitive cells.

7. The GSH of sensitive cells is more easily destroyed by several other reducing substances than is the GSH of nonsensitive cells.

8. The relationship of this phenomenon to drug sensitivity is discussed.

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THE CONCENTRATION IN PLASMA OF POTASSIUM AND CALCIUM DURING TESTOSTERONE MEDICATION

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BETHESDA, MD.

WITH THE TECHNICAL ASSISTANCE OF J. BLAKELY, B.A.

THE finding of reduced serum potassium concentrations, some below 1 mM per liter, in three of about 12 children given methyl testosterone or testosterone proprionate has been reported.[1*] The electrocardiogram did not suggest the presence of hypokalemia and no weakness was noted. The data have been interpreted to indicate that markedly reduced serum potassium concentrations may not in themselves necessarily result in electrocardiographic changes or in clinical symptoms, and that the cell potassium status, the extracellular-cell potassium concentration ratio, or some unknown associated change causes the electrocardiographic changes and paralysis during marked hypokalemia.[1-3] This interpretation is based on the finding that testerone therapy regularly results in the cell accumulation of potassium.[4-7†]

Extracellular potassium concentrations of about 2 to 3 mM per liter may not be associated with overt clinical symptoms, but the absence of any weakness, paralysis, or electrocardiographic disturbance in the presence of serum potassium values below 1.0 mM per liter is very surprising.[8-16] Therefore, as no other data concerning the plasma potassium concentration of young patients given large doses of testosterone have been reported, an attempt was made to confirm the observation that testosterone medication results in hypokalemia. The calcium concentration of plasma was also determined in some patients.

METHODS AND EXPERIMENTAL PROTOCOL

The experiment followed as closely as possible that in which pronounced hypokalemia was reported.[1‡] The subjects, 10 prepubertal children between the ages of 6 and 11 years, were undersized for age. With the exception of one with hypothyroidism and another with Addison's disease the dwarfism was not known to be associated with an endocrine disturbance. In two instances the patients were also under weight for size, indicating the possibility of "nutritional dwarfism."[2]

The patients received 25 mg. of methyl testosterone orally 3 to 4 times a day, one tablet being given on arising, another at bed time, and the other one or two during the day. A 1 to 2 week supply was usually given, with the instruction to call back when the

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*A fourth patient, who received desoxycorticosterone together with methyl testosterone, also showed hypokalemia.

†The retention of potassium and nitrogen during testosterone medication is in proportion to the muscle tissue potassium-nitrogen ratio.[4] It has not been determined whether nitrogen accumulation occurs during testosterone therapy when potassium is excluded from the diet.

‡Personal communication; Dr. N. B. Talbot, Massachusetts General Hospital, Boston, Mass.

medication was about finished. The timing of the return calls indicated that the medication was given regularly. Diet was ad libitum. In all instances the appetite increased markedly, and the body weight increased from 6 to 15 per cent. Except for hoarseness in one patient, no overt undesirable clinical side effects were noted. Blood samples were taken at 9 to 10 A.M. with the patient fasting. Four of the patients were given two courses of testosterone.

Potassium was measured with a flame photometer, with use of an internal lithium standard.[16] Calcium was determined by the method of Clark and Collip.[17]

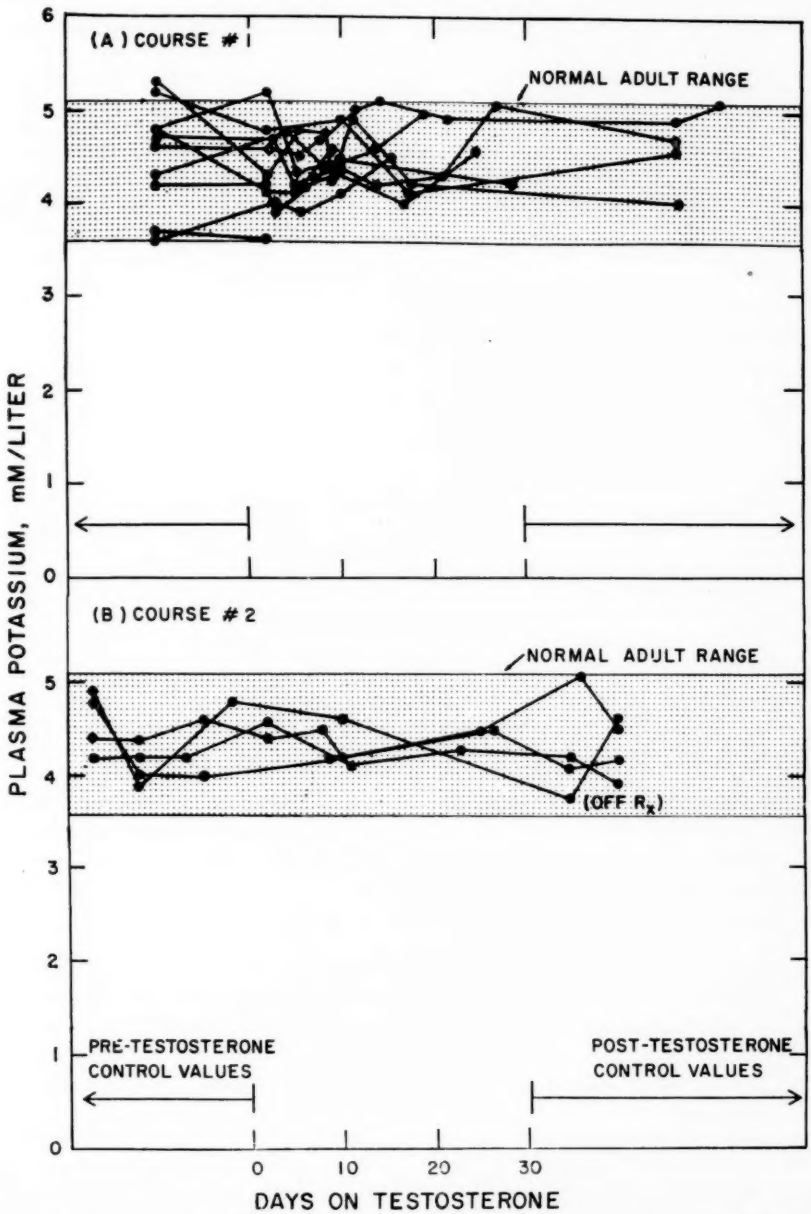

Fig. 1.—The plasma concentration of potassium during testosterone medication.

RESULTS

The concentration of potassium and calcium in the plasma before, during, and following methyl testosterone is shown in Figs. 1 and 2. There is no indication of a systematic change in the concentration of either cation during testosterone medication. The 56 plasma potassium values obtained during testosterone medication fall, with but one exception, in the range of the plasma concentrations seen in the control periods, and in the range described for fasting normal subjects.[3, 18]

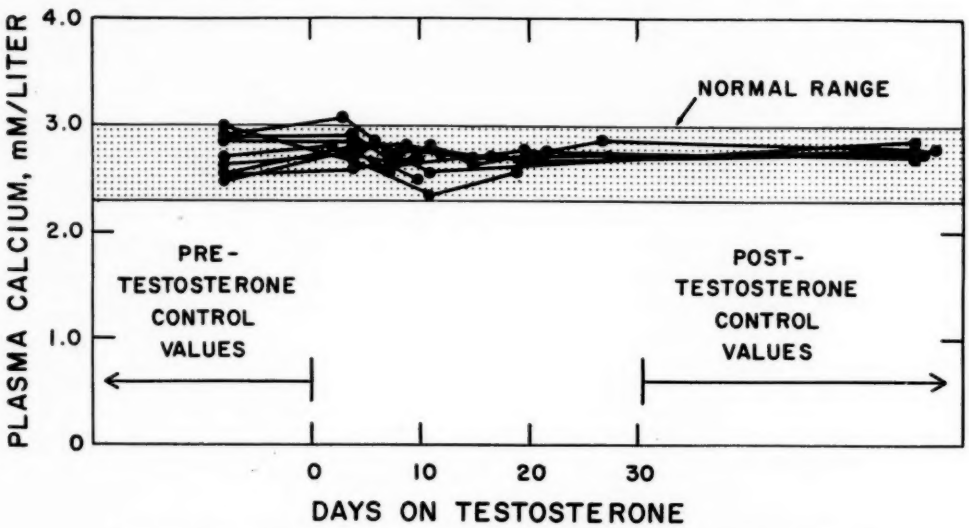

Fig. 2.—The plasma concentration of calcium during testosterone medication.

COMMENTS

The present data, showing no change in the plasma potassium concentration during testosterone medication, are difficult to reconcile with the earlier data indicating the occasional occurrence of marked hypokalemia.[1] Possibly the apparent discrepancy is due to biologic variability, hypokalemia occurring in only a small minority of individuals given testosterone and particularly those given testosterone proprionate, or to change in dietary intake. The subjects in both studies were on an ad libitum diet.

The occurrence of elevated calcium concentrations in patients with osteolytic metastasis given testosterone has been reported.[19-20] The present data confirm the earlier suggestion that little if any alteration in the plasma concentration of calcium occurs in subjects without osteolytic metastasis given large doses of testosterone.[20]

CONCLUSIONS

Ten undersized children were given one or two courses of 75 to 100 mg. of methyl testosterone a day for 30 days. A 6 to 15 per cent increase in body weight occurred. Repetitive analysis of plasma failed to disclose a change in the concentration of potassium or calcium during testosterone medication.

ADDENDUM

We wish to thank Dr. Fuller Albright and Miss Evelyn Carroll, Massachusetts General Hospital, Boston, Massachusetts, for allowing us to see unpublished data, including many serum potassium values, of 4 adult patients, 2 of whom were given testosterone proprionate, one who received methyl testosterone, and one who was given testosterone pellets. A significant change in the serum potassium concentration was not observed in these patients. The balance data of these patients have been reported in detail elsewhere.[4]

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PATHOLOGIC CHANGES IN THE HEARTS AND KIDNEYS OF SENSITIZED RATS EXPOSED TO COLD

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THIS study concerns the hypothesis of Selye[1] that nonspecific stress can cause a variety of diseases which he calls "the adaptation diseases." According to this concept, adaptative mechanisms, especially those involving the adrenal cortical hormones, may go awry during exposure to stress and cause pathologic changes. A number of interesting pathologic changes can be induced in experimental animals by overdosing them with certain steroids, derivatives of natural steroids, and other hormones.[2] Damage induced by hormones is more likely to occur if the experimental animal (rat) has been sensitized by the removal of one kidney and is given an abnormally high intake of sodium chloride.

If Selye's hypothesis as to the etiology of the "adaptation diseases" is correct, it should be possible to cause diseases by the exposure of experimental animals to naturally occurring forms of stress under naturally occurring conditions. It has not been shown that this is true. According to Selye,[3] the sensitized rat exposed to cold may develop hypertension and nephrosclerosis. Other stresses were relatively ineffective. In the present study we have confirmed the occurrence of renal damage in sensitized rats exposed to cold and have found that the accompanying rise in food intake is essential for the production of severe renal damage.

MATERIALS AND METHODS

Rats of the Sprague-Dawley strain were maintained on Archer Dog Pellets until the experiment was begun. Unilateral nephrectomy was performed in all rats. The animals were then given special diets, as described in Table I, with or without added sodium chloride. The diet was packed tightly into individual Franke animal feeding jars, which were weighed every 24 hours. The rats were exposed to cold in a temperature chamber designed by and built under the supervision of the Mechanical Development Division of The Upjohn Company. It was cooled by a sealed refrigeration unit which ran constantly except for automatically controlled defrosting for 15 minutes every 6 hours. The desired temperature was maintained within 1° C. by a thermostatic control of heating units built into the air ducts of the machine. The animals were housed in 12 individual metabolism cages contained within the temperature chamber. Twelve similar cages were used outside the chamber for the maintenance of control rats at room temperature (25° C.). The initial temperature of the chamber was 25° C. This was lowered 2 degrees each day

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for 5 days and then 1 degree every second day until the desired low temperature was reached and then kept constant throughout the remainder of the experiment. In some of the experiments all of the hair was shaved from each cold-exposed rat twice each week.

TABLE I

DRY MIX DIETS* (GM.)		
CONSTITUENT	MEDIUM PROTEIN	MEDIUM CARBOHYDRATE
Cellu flour (Chicago Dietetic Supply)	60	60
Salt mixture (USP XIV)	40	40
Dried yeast (Pabst)	100	100
Wheat germ oil	10	10
Cod-liver oil (Super D, Upjohn)	10	10
Vitamin K (2-methyl-1,4-naphthoquinone)	0.1	0.1
Mazola oil	310	200
Casein (Labco)	500	160
Starch		200
Sucrose		200
Dextrin		190

*Sodium chloride was added to these basal diets according to the section on "Results."

At the end of the experimental period each rat was anesthetized with ether and exsanguinated, and necropsy was performed. Gross pathology was noted, and the heart, kidney, and adrenal glands (Experiments 3 and 4 only) were weighed and fixed in Bouin's fluid. One adrenal from each animal in Experiment 4 was fixed in 10 per cent neutral formalin. Sections of the hearts and kidneys were stained with hematoxylin and eosin and those of the adrenals with Regaud's iron hematoxylin and Masson stain. Frozen sections were made of the formalin-fixed adrenals and stained with sudan black B for lipid. The experiments were done (by D. J. I.) at the University of Chicago, and the tissues were shipped to the University of Michigan for sectioning and study by one of us (B. L. B.). In a few instances tissues either were not fixed at necropsy or were lost through subsequent accident, so that the numbers of rats reported in the tables do not always represent the number stated to be in an experiment.

RESULTS

Experiment 1.—Unilaterally nephrectomized young female rats were given a medium protein diet with added 4 per cent sodium chloride. The initial weights were approximately 75 grams (Table II). Twelve rats were adapted to low temperature and kept at 1° C. for 60 days. These rats were not shaved during exposure to cold. One rat died during this period. Twelve similar rats were kept at room temperature. During the last 7 days at low temperature the average daily food consumption was 15.1 Gm.; at room temperature the average daily food consumption during the last 7 days was 8.7 Gm. As shown in Table II, the controls gained only a little more weight than did the rats exposed to cold. The weights of the heart, kidney, and adrenal glands were greater in the cold-exposed rats than in the controls. There was no significant group difference in weights of the thymus.

Heart: No gross pathology was seen in any heart. Microscopic changes were uniformly negative for the controls and consisted only of hypertrophy of cardiac muscle cells among the cold-exposed rats.

Kidney: The kidneys of the controls showed a few tiny pits and nodules by gross examination. Those from the cold-exposed rats exhibited nodules,

TABLE II. THE EFFECT OF EXPOSURE TO COLD OR ROOM TEMPERATURE ON THE MEAN WEIGHT OF THE BODY AND ORGANS

EXP. NO.	CONDITIONS	GROUP	T°C.	NO. OF RATS	GAIN BODY WT. (GM.)	ORGAN WEIGHTS (MG.) HEART	KIDNEY	THYMUS	ADRENAL
1	Immature females. Initial weight, 75 ± 5 grams. Medium protein diet ad libitum	Cold (not shaved)	1	11	122 ±2.64*	921 ±26.7	1,997 ±141.3	335 ±17.3	74.0 ±2.32
		Control (not shaved)	25	12	126 ±1.50	678 ±11.5	1,257 ±53.2	313 ±14.1	58.0 ±1.78
2	Immature females. Initial weight 62 ± 5 grams. Medium protein diet ad libitum	Cold (shaved)	5	5	111 ±6.5	1,136 ±82.6	2,174 ±102.2	204 ±23.9	68 ±2.3
		Control (not shaved)	25	12	145 ±5.14	689 ±13.9	1,270 ±28.2	287 ±16.4	55 ±1.61
3	Immature females. Initial weight 75 ± 5 grams. Pair-fed but cold-exposed given added sucrose	Cold (shaved)	5	11	99 ±6.04	1,114 ±22.0	1,501 ±42.4	235 ±27.7	59 ±3.0
		Control (not shaved)	25	12	125 ±2.81	644 ±10.2	1,050 ±18.4	226 ±10.1	61 ±1.51
4	Mature young males. Initial weight, 315 ± 5 grams. Medium carbohydrate diet (4% NaCl) ad libitum. Controls, medium carbohydrate diet plus 10% NaCl	Cold (shaved)	3	12	25	1,879 ±68.8	2,742 ±105	205 ±26	68 ±3.09
		Control (not shaved)	25	12	131	1,450 ±72.1	1,962 ±36	322 ±24.7	45 ±1.08

*Standard error.

gray spots, mild mottling, occasional red spots, and, in 2 cases, mild hydronephrosis. Microscopically the kidneys of the cold-exposed rats revealed considerable change, the mean rating being 2.6 as compared with 0.15 for the controls (Table III). A section of one of the kidneys from this group, which typified 6 of 10 rats, was submitted to Dr. Carl V. Weller,* Department of Pathology, University of Michigan, for his analysis which follows: "Cloudy swelling and hyaline droplet degeneration in much of the epithelium of the convoluted tubules. In many tubules the cytoplasm has a radial striated appearance extending throughout the vertical diameter of the cell. This seems to be a precursor of vacuolar degeneration and eventual liquefactive necrosis. Albuminous material, derived in part from fragmentation of cells, is found in the lumina. Many of the collecting tubules are dilated and some of them contain large hyaline casts. There are also glomerular changes. These involve an increase in the volume of the interstitial material of the glomerulus which is swollen and pale staining. There is some evidence of early regeneration of epithelial cells clothing the capillary loops in the interior of the glomerulus. In a few glomeruli there is the formation of epithelial crescents."

TABLE III. MEAN RATING OF PATHOLOGY

EXP. NO.	TREATMENT	NO. RATS	HEART	KIDNEY
1	Cold-exposed	10	0.9* *(0-1)*	2.6 *(2-3)*
	Control	10	0.0	0.15 *(0-1)*
2	Cold-exposed	10	1.8 *(1-3)*	3.3 *(3-4)*
	Control	12	0.0	0.25
3	Cold-exposed	10	1.15 *(1-2)*	1.95 *(0-3)*
	Control	12	0.0	0.33 *(0-1)*
4	Cold-exposed	10	1.5 *(1-2)*	3.15 *(2-4)*
	Control (10% NaCl diet)	12	1.0 *(1)*	0.75 *(0-1)*

*The mean ratings are based on the following scale: Kidney: *0*, no abnormality; *1*, slight focal intertubular inflammation and/or a few hyaline casts in the tubules; *2*, hypertrophy of the tubules and glomeruli and/or a considerable increase in the frequency of hyaline casts; *3*, glomerular pathology, vascular pathology, or considerable increase in interstitial inflammation; *4*, changes listed previously, more severe.

Heart: *0*, no abnormality; *1*, hypertrophy of cardiac muscle cells; *2*, inflammation with hypertrophy of muscle; *3*, necrosis of vessel walls or general inflammation and more intense muscular hypertrophy; *4*, changes listed previously, more severe.

Experiment 2.—Unilaterally nephrectomized young female rats were given a medium protein diet with added 4 per cent sodium chloride. The average initial weight in each group was 62 grams. Twelve rats were adapted to 5° C. and maintained at this temperature for 60 days. These cold-exposed rats were shaved twice each week. Two rats were found dead and 5 rats were found to be dying during exposure to cold. The survival periods were for 1 rat, 12 days; 2 rats, 15 days; 1 rat, 18 days; 1 rat, 27 days; 1 rat, 33 days; and 1 rat, 52 days. Five rats survived for 60 days. Twelve similar rats were kept at room temperature. During the last 7 days at low temperature the average daily food consumption for the 5 survivors was 16.9 Gm.; at room temperature the average daily food consumption during the last 7 days was 8.2 Gm. The data on the weights of the body and organs are in Table II. The rats exposed to cold gained less weight, but there was enlargement of the heart, kidney, and adrenals. There was some suppression in weight of the thymus among the cold-exposed rats.

*We wish to express our sincere appreciation to Dr. Carl V. Weller for his helpful analysis of some of our sections.

Heart: No gross pathology was seen in the hearts of any of the control rats. The hearts of 2 rats exposed to cold for 60 days had white spots on the surface. In 5 of the 10 cold-exposed rats whose tissues were sectioned, the only alteration observed in the heart was hypertrophy of the muscle cells. In the remainder more significant change was observed. In 2 animals there were areas of "Patchy Zenker's hyaline degeneration with changes approaching those of simple necrosis" (C. V. W.). In 2 cases, rare fibrinoid necrosis was observed in the wall of small blood vessels. There were occasional foci of fibroblastic proliferation, especially around blood vessels.

Kidney: Gross examination showed a few tiny pits and nodules on the kidneys of the control rats. Among the rats exposed to cold the gross damage observed on the surface of the kidney ranged from the presence of many tiny nodules to rough, nodular, and mottled irregularities. Some red spots and gray patches were noted. Microscopically, the kidneys of all of the cold-exposed rats in this experiment were damaged severely. The tubular epithelium was altered in a manner similar to that in Experiment 1. In one of these specimens "there are numerous dilated tubules in some of which there are large hyaline casts. There are patchy areas in the cortex which are interpreted as due to relative ischemia short of complete infarction. Glomerular lesions are more marked than in the test rat of Experiment 1. In certain glomeruli Bowman's capsular space is completely obliterated by heavily eosinophilic material which is almost hyaline but which does show a slight fibrillar pattern concentric with the periphery of the glomerulus. Such glomeruli appear to be nonfunctioning. Other glomeruli show only some moderate increase in epithelial cells" (C. V. W.). "Moderate cloudy swelling of the interstitial substance within the glomeruli" (C. V. W.) was noted in a control rat.

Experiment 3.—The conditions in Experiment 3 were identical with those of Experiment 2, except that the intake of the medium protein diet with added 4 per cent sodium chloride in the cold-exposed rats was limited to the amounts eaten by the controls (8.5 Gm. daily for each rat). The cold-exposed rats were permitted to compensate for their caloric deficit by drinking a solution of 10 per cent sucrose and eating dry sucrose which was placed in their cages each night. The cold-exposed rats ate all of the medium protein diet available to them and consumed the solution of 10 per cent sucrose in quantities which frequently exceeded 100 ml. per rat per day. One cold-exposed rat died on the thirty-seventh day. The 11 survivors appeared to be in excellent condition. The data on body and organ weights (Table II) show that the cold-exposed rats gained less weight than the controls. There was enlargement of the heart and kidney and little change in the average weight of the adrenals and the thymus. However, one rat showed extensive atrophy of the thymus.

Heart: No gross pathology was seen in any heart. Sections of the hearts from the cold-exposed rats uniformly showed myocardial hypertrophy without any other significant change (Figs. 1 and 2).

Kidney: Kidneys from the control rats showed either no gross pathology or minimal damage consisting of a few nodules and pits. Among the cold-exposed rats the gross pathology was slightly more severe than in the controls,

but was less than that observed among the cold-exposed rats in Experiments 1, 2, and 4. Microscopically the kidneys were altered less by exposure to cold in this experiment than in any of the others. In general, the tubules and glomeruli were enlarged as compared with those of the controls and the frequency of intratubular hyaline casts was increased. In one specimen, there was a "slight increase in epithelial nuclei in the glomeruli and possibly some regeneration of epithelium clothing the capillary loops" (C. V. W.).

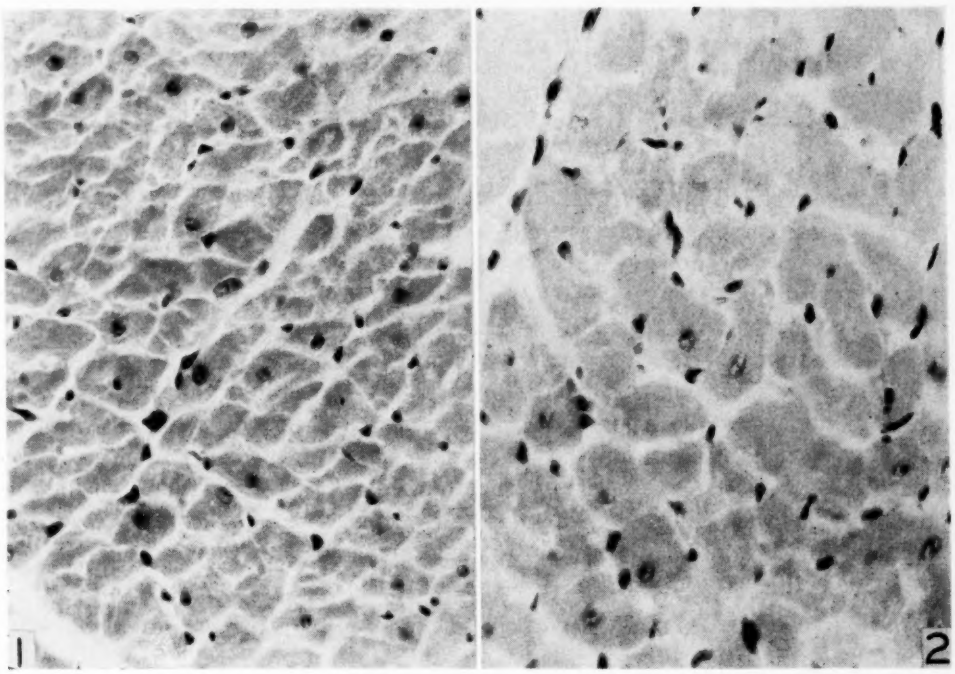


Fig. 1.—Heart. Control rat for that illustrated in Fig. 2. (×285; reduced ⅕.)
Fig. 2.—Heart. Cold-exposed rat from Experiment 3. Muscle cells are hypertrophied. (×285; reduced ⅕.)

Adrenals: As compared with the controls (Fig. 9), the cortical zona glomerulosa of the cold-exposed rats was reduced in width; its cells were smaller, less vacuolated, and contained denser cytoplasm. The zone was demarcated sharply from the large, vacuolated cells of the outer zona fasciculata. Beyond slight cellular hypertrophy, no changes were observed in the zona fasciculata by exposure to cold. The zona reticularis was modified by exposure to cold in an irregular manner. A band of slightly enlarged vacuolated cells near the medulla was separated from the zona fasciculata by a narrow region of cells which resembled those of the zona reticularis in the controls. The latter were reduced in size and interspersed with sinusoids which possessed thickened walls. Exposure to cold did not alter the medulla.

Experiment 4.—Mature young male rats (initial average weight of 315 grams) were subjected to unilateral nephrectomy and were given a medium carbohydrate diet to eat ad libitum. Since the cold-exposed rats showed less damage when their intake of sodium chloride was limited to that of rats kept

at room temperature (Experiment 3), Experiment 4 was intended to test the possibility that the high load of sodium chloride rather than cold per se caused severe renal damage. The cold-exposed (3° C. for 60 days) rats received the diet with added 4 per cent sodium chloride; the rats kept at room temperature received the same diet with added 10 per cent sodium chloride. All of the rats kept at room temperature survived. Two of the cold-exposed rats died on the fifty-first day. The average daily food intake for the rats kept at room temperature was 16.3 Gm. per rat; for the cold-exposed rats which survived, it was 29.1 Gm. per rat. The data on body and organ weights (Table II) show that the cold-exposed rats gained much less than the rats kept at room temperature and that the hearts, kidneys, and adrenal glands were enlarged in the cold-exposed rats. There was atrophy of the thymus in 3 cold-exposed rats with some suppression of thymic weight in others of this group.

No abnormal behavior or symptoms of disease were noted among the rats kept at room temperature other than a temporary hematuria in three animals. "Fits" of squealing and biting were occasionally noted among the cold-exposed rats. Self-inflicted wounds were found on the tail and hind legs of 3 rats. This behavior sometimes occurred in the absence of any obvious external stimulus, but it could be elicited by handling the rat. The "fits" did not represent true convulsions but the rat behaved as though it were in pain. During intervals between "fits" the rats did not appear to be hyperirritable. One rat suddenly became comatose on the fifty-eighth day at 3° C. It was not cold or otherwise overtly ill. At necropsy the following day it was found to have a massive hemorrhage into the left cerebral cortex. One rat developed an extensive ocular hemorrhage. Temporary hematurias developed in 4 cold-exposed rats. One rat developed a badly swollen hind foot.

Heart: The hearts of the rats kept at room temperature on the 10 per cent sodium chloride diet showed no gross pathology. Those from 5 of the rats exposed to cold possessed white spots on the surface. Microscopically, the cardiac muscle cells were regularly hypertrophied ofter exposure to cold. There was occasional fibroblastic proliferation around small blood vessels and some increase in the frequency of Anitschkow myocytes.

Kidney: Minimal gross damage to the kidneys was observed in the rats kept at room temperature on the 10 per cent sodium chloride diet. There was one case of hydronephrosis but the surface of the kidney was normal. Two rats had tiny nodules on the surface of the kidney. Among the cold-exposed rats, two kidneys showed only a few tiny nodules on the surface but the remaining kidneys were mottled and usually rough and nodular. In one kidney the juxtapelvic zone was patchy yellow.

Microscopically, although there was some variation in the intensity of renal pathology after subjection to cold, most of the rats in this group exhibited considerable damage. In one of these specimens, "[the] kidney shows severe changes of subchronic progressive glomerulonephritis. There are both vascular and parenchymal lesions. Taken by themselves the vascular lesions are like those of arteriolosclerotic nephropathy with obliteration of arterioles and much

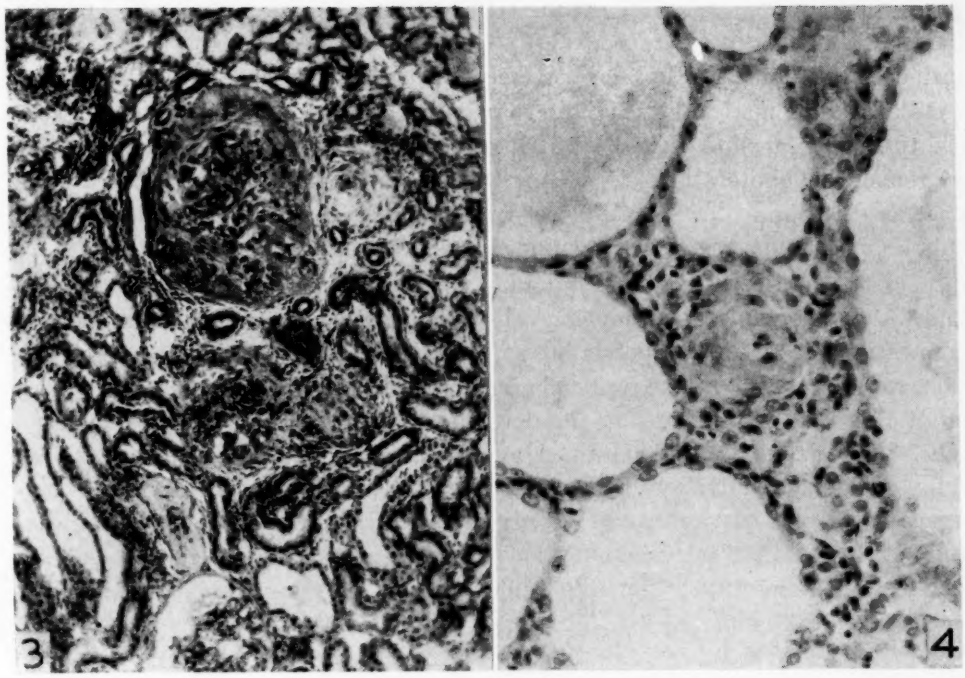


Fig. 3.—Kidney of cold-exposed rat (Experiment 4). One glomerulus is partially hyalinized. Beneath the glomerulus is a sclerotic arteriole. (×95; reduced ⅕.)

Fig. 4.—Kidney of cold-exposed rat (Experiment 2). Necrosis of the wall of a small vessel with cellular infiltration around it. (×320; reduced ⅕.)

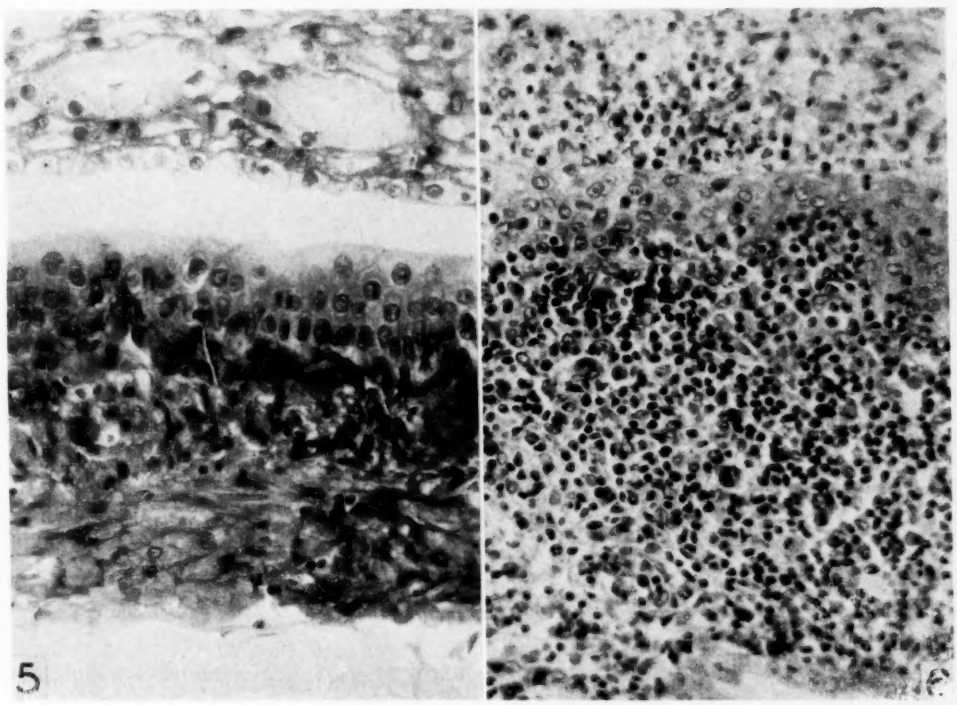


Fig. 5.—Pelvic wall. Control for rat illustrated in Fig. 4. (×340; reduced ⅕.)

Fig. 6.—Pelvic wall. Cold-exposed rat from Experiment 4. A lymphocyte-producing area with mononuclear cells infiltrating through the transitional epithelium into the pelvic lumen above. (×340; reduced ⅕.)

intimal proliferation (Fig. 4). In the glomeruli there are many thrombosed capillaries and many glomeruli are partially obliterated by adhesions between the tufts and capsules (Fig. 3). This results in the presence of various degrees of glomerular obliteration. The renal tubular epithelium shows hyaline droplet degeneration and there is intensely eosinophilic hyaline material in many tubules of the type suggesting hyaline albuminous casts''(C. V. W.).

In five of the 10 cold-exposed rats, the pelvic wall was inflamed (Figs. 5 and 6). Neutrophils and lymphoid cells were infiltrating from the subepithelial connective tissue into the pelvic lumen. Extensive areas of lymphocyte formation were present. These were characterized by the occurrence of frequent mitotic figures, of all stages in the development of lymphocytes, and of plasma cells. The pelvic lumen contained aggregations of neutrophils, mononuclear cells, desquamated transitional epithelium, and tissue debris. The most significant evidence of vascular pathology occurred in Experiment 4. It is probably pertinent that in the kidneys of this experiment inflammatory changes also were most evident. Thus, the appearance of vascular pathology may have been related to the factor(s) which elicited the inflammatory reaction.

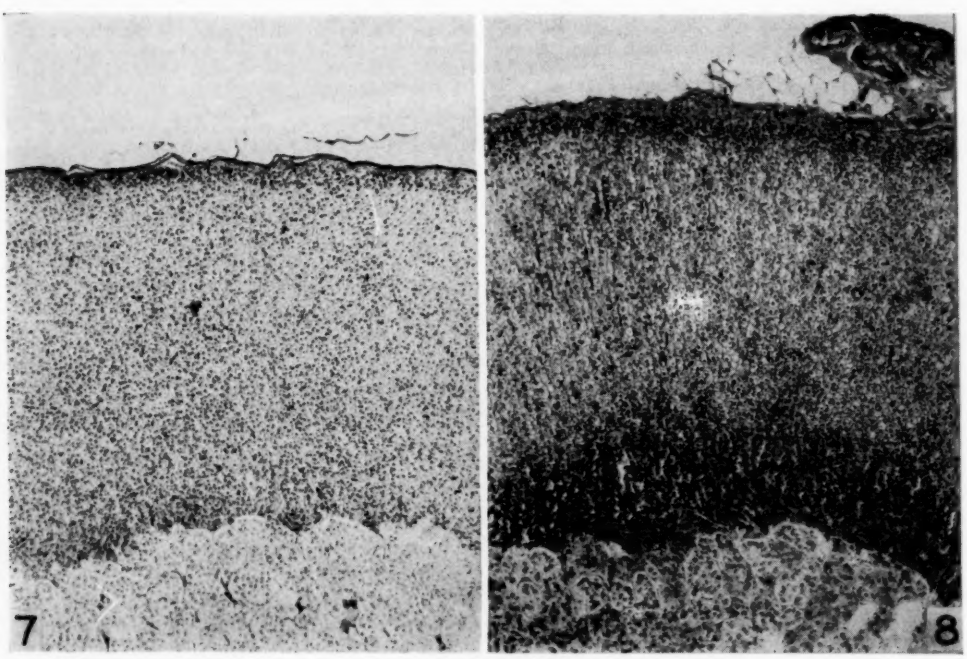
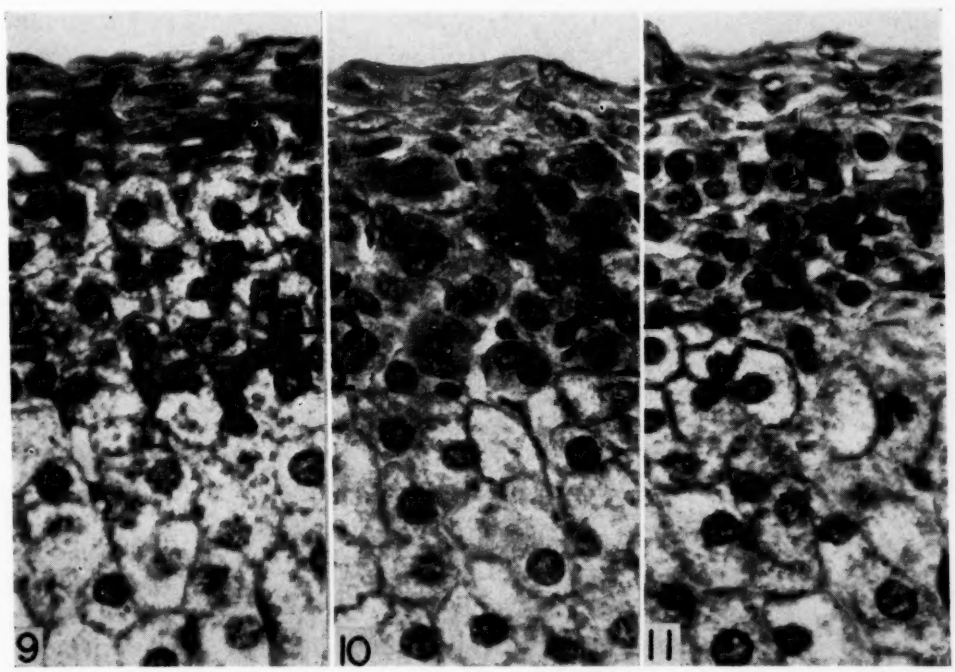


Fig. 7.—Adrenal cortex and medulla from a control rat in Experiment 4. (×75; reduced ⅕.)

Fig. 8.—Adrenal cortex and medulla from a rat in Experiment 4 which was exposed to cold. The zont reticularis is wider than in the control (Fig. 7). (×75; reduced ⅕.)

Adrenals: As compared with the control rats of Experiment 3 which received 4 per cent NaCl in their diet (Fig. 9), the controls of Experiment 4 which received 10 per cent NaCl (Fig. 10) exhibited a somewhat atrophic zona

glomerulosa with cellular characteristics similar to those described for the cold-exposed rats of Experiment 3). Practically no sudanophilic lipid was present. In the rats which were exposed to cold but received less salt (4 per cent NaCl), some lipid was demonstrated in the zona glomerulosa by sudan staining but its cells were smaller than was true of the controls in Experiment 3 (Fig. 11). The zona fasciculata showed little change except for the appearance of somewhat larger lipid vacuoles. The zona reticularis was wider after exposure of rats to cold and possessed smaller, lipid-poor cells (Figs. 7 and 8). In the medulla, exposure to cold induced a general reduction in size of cells and in circumscribed areas, a condensation of cytoplasm which seemed to extend out from the cytocentrum into the peripheral portion of the cell.



Figs. 9-11.—These figures illustrate the zona glomerulosa and outer portion of the zona fasciculata of the adrenal cortex. Lines represent the approximate boundary between the two zones.

Fig. 9.—From a control rat in Experiment 3. (×720; reduced ⅕.)

Fig. 10.—From a control rat (Experiment 4). The cells of the zona glomerulosa are slightly reduced in size as compared with those in Fig. 9 and contain fewer vacuoles, which indicates a reduced amount of lipid. (×720, reduced ⅕.)

Fig. 11.—From a rat exposed to cold (Experiment 4). The zona glomerulosa is more atrophic than that shown in Fig. 10. (×720; reduced ⅕.)

DISCUSSION

These experiments support the claim by Selye[3] that exposure of the "sensitized" rat to cold will cause renal pathology. The cold-exposed rat must increase its caloric intake above normal to meet increased energy requirements or it will die. The increased dietary load, especially of sodium chloride, would seem to be an essential condition for the production of severe renal damage in

the cold-exposed rat. Evidence supporting this interpretation was found in Experiment 3, in which the intake of the high sodium chloride diet by the cold-exposed rats was limited to that eaten by rats at room temperature. However, it might also be claimed that the sucrose available to the cold-exposed rats had some protective influence on the kidney.

The increased renal damage in the cold-exposed rats is not caused solely by the increased sodium chloride intake. In Experiment 4, the feeding of a 10 per cent sodium chloride diet to rats at room temperature represented a higher total sodium chloride intake than did the 4 per cent sodium chloride diet eaten by the cold-exposed rats. The renal damage among the rats at room temperature was increased only a little and that of the cold-exposed rats was severe. Also, in Experiment 3, exposure to cold caused a mild increase in renal pathology above that of rats at room temperature on the same sodium chloride load.

As noted by Selye,[3] the myocardial damage among rats exposed to cold was usually not severe. The pattern of pathologic changes in the hearts and kidneys does not closely resemble the pattern of changes induced by 11-desoxycorticosterone and adrenal cortical extract in sensitized rats,[4] although there are some features in common. Nephrosclerosis was observed in some of the cold-exposed rats (Experiment 4), but most of the lesions represented nephritis.

The occurrence of a cerebral hemorrhage in one of the cold-exposed rats is of interest. Selye[3] reported a high incidence of hypertension among sensitized rats exposed to cold but no data were published.

The role of the adrenal cortical hormones in causing the renal and myocardial pathology of sensitized rats exposed to cold has not been shown. In these experiments mild hypertrophy of the adrenal glands was observed in Experiments 1, 2, and 4. In most rats the thymus failed to atrophy during exposure to cold. The thymus is a sensitive indicator of severe hyperadrenocorticalism.

The histology of the zona glomerulosa is pertinent to a consideration of the induction of pathology by stress. Swann[5] and Deane and Greep[6] have postulated that salt-regulating steroids are secreted by this region. The administration of one of these steroids, desoxycorticosterone, induces vascular pathology in rats.[1] Most of the changes which we observed in the zona glomerulosa can be explained on the basis of the high concentration of dietary Na, which has been shown to induce regression of this region.[7] However, in Experiment 3, where the intake of Na was equilibrated between controls and cold-exposed rats by pair-feeding, other factors may have been operative in causing the marked reduction in size of the cells of the zona glomerulosa after exposure to cold. In Experiment 4 both groups received high dietary NaCl and in both, the zona glomerulosa exhibited partial atrophy, lipid being practically absent in the controls which had received the higher dietary load of NaCl (10 per cent). Thus, no evidence was obtained which clearly indicated an acceleration in secretion of a salt-active steroid by the zona glomerulosa. On the contrary, the histologic changes observed suggest that the secretory activity of the zona glomerulosa was suppressed in the cold-exposed rats.

Sellers and You[8] have reported cardiovascular lesions in normal rats which were fed a normal diet during exposure to cold for 10 to 18 months. Eighteen of 29 rats had stainable lipid in the coronary arteries. No fat was demonstrated in the coronary vessels of 27 control rats. When blood pressure was measured directly under light ether anesthesia, all values for the cold-exposed rats were within normal range. By the indirect method, 3 cold-exposed rats were "hypertensive," although the average for the group was 143 ± 8 mm. as compared to 130 ± 5 mm. for the controls. Pathologic changes in the kidneys consisted of: "(1) degeneration of tubular cells with deposition of fat in the cells; (2) dilatation of tubules with appearance of casts which often were fatty; (3) appearance of fat in cells of the glomerular capsule or in the basement membrane adjacent to glomerular capillaries; (4) ischaemia and deposition of hyaline material in loops of the glomerular capillaries; and (5) no major changes or deposition of fat in renal arterioles or arteries."

The production of pathologic changes, resembling to some extent diseases which occur in man, by exposure of the rat to cold are of interest. However, the conditions which seem to be essential for the production of damage are not likely to be naturally occurring. The role of hormones in causing the pathology has not been established. It remains to be shown that exposure to nonspecific forms of stress can cause diseases in either laboratory animals or in man.

SUMMARY

Rats of the Sprague-Dawley strain were sensitized by removal of one kidney and the feeding of a diet containing 4 per cent sodium chloride. In Experiments 1 and 2, immature female rats developed severe renal damage and mild myocardial changes when exposed to cold for 60 days. The food intake of the cold-exposed rats was greatly increased above the amounts eaten by similar rats kept at room temperature. In Experiment 3, immature female rats were sensitized and all were given equal amounts of the 4 per cent sodium chloride diet to eat. The cold-exposed rats were permitted to meet their extra caloric needs by eating sucrose. Under these conditions the cold-exposed rats showed somewhat greater renal damage than did similar rats at room temperature, but the damage was mild as compared to the pathology of similar cold-exposed rats eating the high salt diet ad libitum. In Experiment 4, mature sensitized male rats were permitted to eat the 4 per cent sodium chloride diet; similar rats at room temperature ate a 10 per cent sodium chloride diet. Although the total sodium chloride intake was higher in the rats kept at room temperature, the renal and myocardial damage was mild as compared to the severe damage among the cold-exposed rats. One of the rats had a cerebral hemorrhage during exposure to cold. The principal renal lesions were inflammatory in nature. Nephrosclerosis was seen in a few instances.

It is concluded that the pathologic changes which appear in the sensitized rat exposed to cold are dependent in part upon the increase in food-intake, especially of sodium chloride. However, a 10 per cent sodium chloride diet did

not cause severe damage in rats kept at room temperature. The experimental conditions used here are not naturally occurring. The role of the endocrine system in the production of damage under these conditions is not known.

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THE PLASMA ERYTHROPOIETIC-STIMULATING FACTOR IN MAN

Observations on Patients With Polycythemia Vera and Secondary Polycythemia

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THE mechanisms regulating erythropoiesis have long been the subject of speculation.[1, 2] Since its existence was first postulated by Carnot and Deflandre[3] in 1906, the role of a humoral factor or factors has generated considerable interest.[4-7] The recent demonstration that protein-free extracts of "anemic" rabbit plasma are capable of stimulating erythropoiesis in the normal rat[8-10] has greatly facilitated investigation of this factor.

It is the purpose of this report to describe studies involving the assay of human nonprotein plasma extracts from patients with polycythemia vera and secondary polycythemia for their ability to stimulate erythropoiesis in the rat. In his recent monograph, Lawrence[11] states that Contopoulos and co-workers have extracted a plasma factor from patients with polycythemia vera capable of stimulating erythropoiesis in the rat, but no details are given.

MATERIALS AND METHODS

Human plasma was obtained from two patients with polycythemia vera and two patients with secondary polycythemia (see case reports below). The plasma was immediately frozen and so kept until the protein-free plasma extracts (hereafter designated as PFPE) were prepared according to the method originally described by Borsook and associates,[8] plus precipitation with perchloric acid as previously outlined by us.[10] Normal human plasma was procured from blood bank donors with normal hemoglobin values.

Seventy-four female Wistar-strain rats, weighing approximately 160 grams, were divided into six groups. Each rat received 18 daily subcutaneous injections over a three-week period (Sundays excepted) in amounts equivalent to 2 per cent of their body weight. Twelve rats were injected with PFPE-polycythemia vera (R. B.), 7 with PFPE-polycythemia vera (J. P.), 8 with PFPE-secondary polycythemia (T. N.), 7 with PFPE-secondary polycythemia (I. J.), and 16 received normal human PFPE. Twenty-four rats were given Ringer's solution.

Baseline hematology studies consisted of hemoglobin, hematocrit, erythrocyte, and reticulocyte determinations. The former 3 were repeated at weekly intervals and the latter twice weekly for 4 weeks, a period lasting one week after the injections were discontinued. Hemoglobins were done by an oxyhemoglobin photoelectric colorimeter method, hematocrits by a microhematocrit technique, and red blood cells were enumerated by hemacytometer.

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Reticulocyte percentage was determined by counting the number of reticulocytes per 1,000 red cells on dried brilliant cresyl blue coverslip films counterstained with Leishman's stain. Blood for these studies was obtained from the tail vein of the rat.

At the conclusion of the injection period, 4 rats from each of the first 4 groups, 8 animals receiving normal human PFPE, and 14 from the Ringer's solution group were killed and femoral marrow was secured and examined according to the method previously described by us.[10] The remaining rats were used to evaluate the rapidity of return to normal levels following discontinuation of the injections.

All rats were weighed twice weekly and the dosage of the materials given by subcutaneous injection was adjusted accordingly.

CASE REPORTS

R. B.—UMH 830179: A 73-year-old white night watchman was first seen at the Simpson Memorial Institute on December 15, 1955. He had been apparently well until approximately one month prior to admission when he developed an episode of dyspnea of relatively short duration. He noted similar recurrent attacks and consulted his physician in early December and was digitalized. Hepatosplenomegaly was noted at this time and examination of his blood revealed increased erythroid values. He denied exertional dyspnea, orthopnea, paroxysmal nocturnal dyspnea, or chest pain but had noted intermittent ankle edema for approximately one year. He had never been phlebotomized. System review and past and family history were otherwise noncontributory.

Physical examination demonstrated plethora, arteriosclerotic changes of the retinal vessels, cardiomegaly with numerous premature contractions, hepatic and splenic enlargement extending 10 and 15 cm. below the right and left costal margins, respectively, and moderate ankle and pretibial pitting edema. The blood pressure was 140/80.

The hemoglobin was 18.7 Gm. per cent; R. B. C.: 8.5 million; hematocrit: 63 volumes per cent; and W. B. C.: 16,150. The differential count revealed band neutrophils: 32; segmented neutrophils: 52; lymphocytes: 3; monocytes: 7; eosinophils: 3; and basophils: 3 per cent. Platelets were within the normal range and the reticulocytes were 1.5 per cent. The total serum bilirubin was 1.2 mg. per cent with 0.8 mg. per cent in the indirect fraction. Other liver function studies were normal. The serum uric acid was 10.5 and the nonprotein nitrogen 83 mg. per cent, respectively. A chest x-ray showed generalized cardiomegaly, pulmonary congestion, and bilateral pleural effusions. An electrocardiogram revealed T-wave changes attributable to digitalis and no other abnormalities.

Sternal marrow aspiration yielded a hypercellular specimen with a relative and absolute increase in erythropoiesis. The E:G ratio was 3:2. Granulopoiesis was active and orderly. Megakaryocytes were numerous and there was no evidence of leukemia or malignant infiltration in the specimen.

A diagnosis was made of polycythemia vera. Approximately 2,000 ml. of blood was removed over a period of 4 days and the patient was then given radioactive phosphorus.

J. P.—UMH 835356: A 58-year-old white male service station attendant was admitted to the University Hospital on February 22, 1956. He stated that his complexion had been ruddy for at least 25 years. He received chiropractic manipulation on February 20, 1956, because of low back pain which had been present intermittently for many years. Following this, he noted increased low back and left hip and thigh pain and consulted his physician, who found increased erythroid values. No phlebotomies were performed. His history was otherwise noncontributory.

Physical examination revealed a well-developed, well-nourished plethoric male with a blood pressure of 148/90. Funduscopic examination showed venous engorgement. The heart and lungs were not remarkable. The liver and spleen were each palpable 7 cm. below their respective costal margins. There were tenderness, swelling, and extensive ecchymoses over the region of the left hip and thigh.

The hemoglobin on admission was 21.6 Gm. per cent, hematocrit: 72 volumes per cent, and the W. B. C.: 16,900. The differential revealed myelocytes: 1, metamyelocytes: 1, band neutrophils: 13, segmented neutrophils: 72, lymphocytes: 5, monocytes: 7, and basophils: 1 per cent. Platelets were increased in number and the reticulocytes were 2 per cent. Moderate polychromasia was evident. The total serum bilirubin was 2.4 mg. per cent

with 2.1 mg. per cent as the indirect form. Serum uric acid was 3.4 and the NPN 50 mg. per cent, respectively. Chest x-ray was normal and spine films showed scoliosis of the lumbar spine with hypertrophic spurring. The blood volume by the RISA method was 8,750 ml. with an estimated volume based on height of 5,500 ml.

The sternal marrow was hypercellular with a relative and absolute increase in erythropoiesis which was normoblastic in type with many early forms. The E:G ratio was 1:1. Granulopoiesis was orderly. Megakaryocytes were increased in number with active platelet budding.

A diagnosis of polycythemia vera was made and the patient was phlebotomized down to a hematocrit of 57 volumes per cent in a period of 3 days and was then given radioactive phosphorus.

T. N.—UMH 804296: A 20-year-old white male student was initially seen at the University Hospital in February, 1955. He gave a history of cyanosis and exertional dyspnea since birth without signs of frank congestive failure.

Physical examination revealed a well-developed, well-nourished cyanotic white male with marked clubbing of his fingers and toes. The blood pressure was 146/130 and the pulse was 134. The heart was enlarged to the anterior axillary line with a systolic outthrust along the left sternal border. There were no significant murmurs, but a presystolic extrasound was present. There was no peripheral edema or evidence of increased venous pressure and the remainder of the physical examination was not remarkable. The liver and spleen were not palpable.

The hemoglobin was 24.2 Gm. per cent, hematocrit: 85 volumes per cent, and the W. B. C.: 9,100 with a normal differential. Platelets were within the normal range.

The total serum bilirubin was 1.9 mg. per cent with 1.4 mg. per cent as the indirect form. An electrocardiogram showed sinus tachycardia, unusual QRS complexes in the limb leads, large P waves in lead II, and the unipolar chest leads were interpreted as showing probable right ventricular hypertrophy. A chest x-ray demonstrated cardiomegaly which was predominately right ventricular and slight prominence of the pulmonary artery with the hilar and peripheral vessels appearing essentially normal.

Serial phlebotomies were instituted in February, 1955, in an attempt to maintain the hematocrit at levels of about 60 to 65 volumes per cent. The plasma used in the erythropoiesis experiments was derived from blood obtained between February and May. The patient noted increased weakness and exertional dyspnea over the subsequent months. Cardiac catheterization had been scheduled but the patient was admitted to the hospital in acute respiratory distress on June 20, 1955, and expired almost immediately.

Necropsy confirmed the diagnosis of congenital heart disease with secondary polycythemia. There was a patent interventricular septal defect with overriding of the aorta and a narrowing of the pulmonary artery just above the valve.

I. J.—UMH 835350: A 69-year-old white man, employed as a meat cutter, was admitted to the University Hospital on February 22, 1956. He fell and fractured several ribs on December 31, 1955, at which time he was first found to be polycythemic, although he stated that his complexion had been ruddy for at least 50 years. He denied any other symptoms except for some recent intermittent claudication in the right leg. He had noted 2 to 3 nosebleeds per year but denied all other forms of abnormal bleeding and his family history was noncontributory in this regard. He had had no therapeutic withdrawal of blood. A right upper lung abnormality had been present and apparently unchanged for 18 years and was thought to represent tuberculous scarring.

Physical examination revealed a well-developed, well-nourished white man with a reddish-blue plethoric appearance. The blood pressure was 130/80. There was marked clubbing of the fingers and toes. There were numerous telangiectasia over the trunk. Funduscopic examination showed arteriosclerotic changes plus venous engorgement. Examination of the heart and lungs was not remarkable. The liver was palpable 4 cm. below the right costal margin but no splenic enlargement could be demonstrated. The dorsalis pedis and posterior tibial pulses were absent on the right.

The hemoglobin was 20.4 Gm. per cent, R. B. C.: 6.8 million, hematocrit: 70.5 volumes per cent, and W. B. C.: 6.350 with a normal differential. Platelets were present

in normal numbers. The total serum bilirubin was 0.7, NPN: 39, and serum uric acid 5.1 mg. per cent, respectively.

A sternal marrow specimen was cellular with a relative and absolute increase in erythropoiesis which was normoblastic with a large number of early forms. The E:G ratio was 1:1. Granulopoiesis was orderly but not hyperactive and megakaryocytes were present in normal numbers. Blood volume by the RISA method was 7,676 ml. with an estimated volume based on height of 5,400 ml. The resting arterial oxygen saturation was 73 per cent, after the breathing of 100 per cent oxygen it was 84 per cent, and after exercise for 3 minutes breathing room air it was 73 per cent.

Chest x-ray revealed a right upper lung field parenchymal abnormality which was seen to pulsate on fluoroscopy. Angiocardiography demonstrated the abnormality to be an extensive arteriovenous malformation.

This patient was phlebotomized over a period of 3 days and discharged with the diagnoses of probable hereditary hemorrhagic telangiectasia, pulmonary arteriovenous fistula of the right upper lung, and secondary polycythemia.

RESULTS

Protein-free extracts of human plasma from patients with both polycythemia vera and secondary polycythemia contain a factor capable of stimulating erythropoiesis in the normal rat. This was demonstrated by erythrocytosis (Fig. 1 and Table I) and reticulocytosis (Fig. 2), plus the increased marrow erythropoietic activity shown in Fig. 3 and Table II. Although both the

TABLE I. STANDARD DEVIATION IN THE ERYTHROCYTES (MILLIONS PER CUBIC MILLIMETER)

GROUPS	BASELINE		1 WEEK		2 WEEKS		3 WEEKS*		4 WEEKS	
	MEAN	SD	MEAN	SD	MEAN	SD	MEAN	SD	MEAN	SD
PFPE—Normal human	8.23	0.63	8.48	0.60	8.35	0.39	8.28	0.36	9.07	0.66
Ringer's solution	8.30	0.64	8.01	0.45	7.97	0.67	8.07	0.71	8.50	0.57
PFPE—polycythemia vera (R. B.—UMH 830179)	8.28	0.34	10.66	0.25	11.35	0.47	12.35	0.52	8.83	0.79
PFPE—polycythemia vera (J. P.—UMH 835356)	8.36	0.72	11.04	0.46	12.08	0.61	12.49	0.80	8.62	1.05
PFPE—secondary polycythemia (T. N.—UMH 804296)	7.38	0.73	10.60	0.47	12.09	0.68	12.85	0.26	8.33	0.35
PFPE—secondary polycythemia (I. J.—UMH 835350)	8.49	0.43	11.74	0.40	12.61	0.35	12.88	0.28	8.31	0.26

*Injections discontinued.

TABLE II. AVERAGE MARROW GRANULOCYTIC AND ERYTHROCYTIC ELEMENTS EXPRESSED AS PER CENT OF ALL CELLS

GROUPS	TOTAL GRANULOCYTIC	TOTAL ERYTHROCYTIC	RUBRIBLASTS	PRORUBRICYTES	RUBRICYTES	METARUBRICYTES
PFPE—normal human	65.8	16.3	0.1	0.3	2.2	13.7
Ringer's solution	63.1	18.2	0.1	0.4	2.2	15.5
PFPE—polycythemia vera (R. B.—UMH 830179)	45.2	41.5	1.0	2.4	7.1	31.0
PFPE—polycythemia vera (J. P.—UMH 835356)	43.9	43.6	1.6	2.7	8.6	30.7
PFPE—secondary polycythemia (T. N.—UMH 804296)	44.7	41.8	1.1	2.5	8.1	30.1
PFPE—secondary polycythemia (I. J.—UMH 835350)	45.6	43.3	1.2	2.8	11.5	27.8

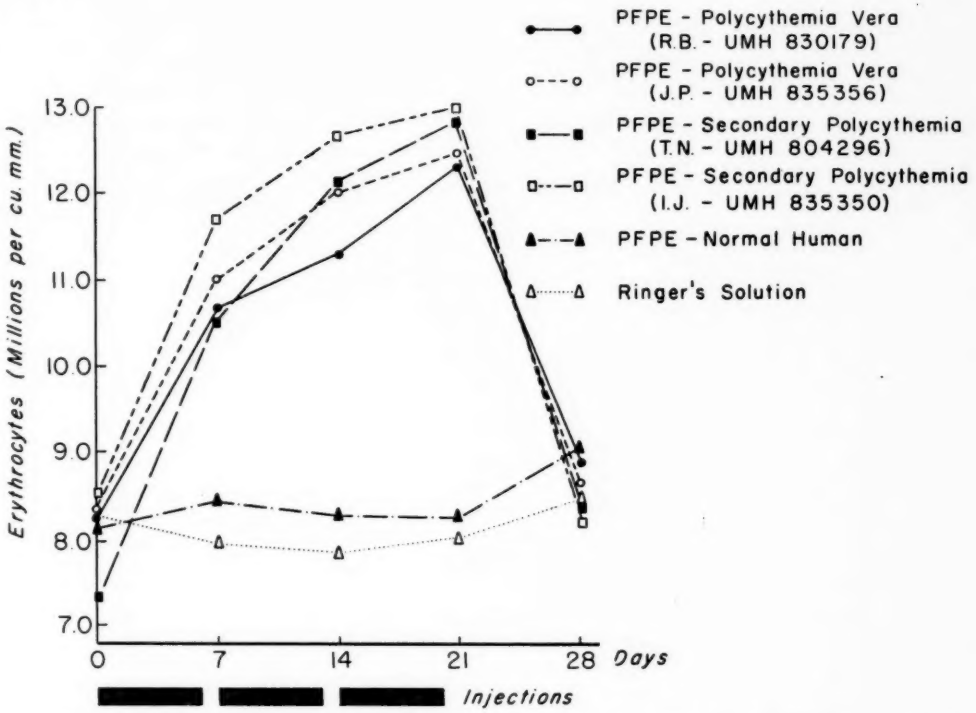

Fig. 1.—Erythrocytosis in rats injected with plasma extracts from patients with polycythemia vera and secondary polycythemia. Mean of erythrocyte counts of rats receiving the above described materials.

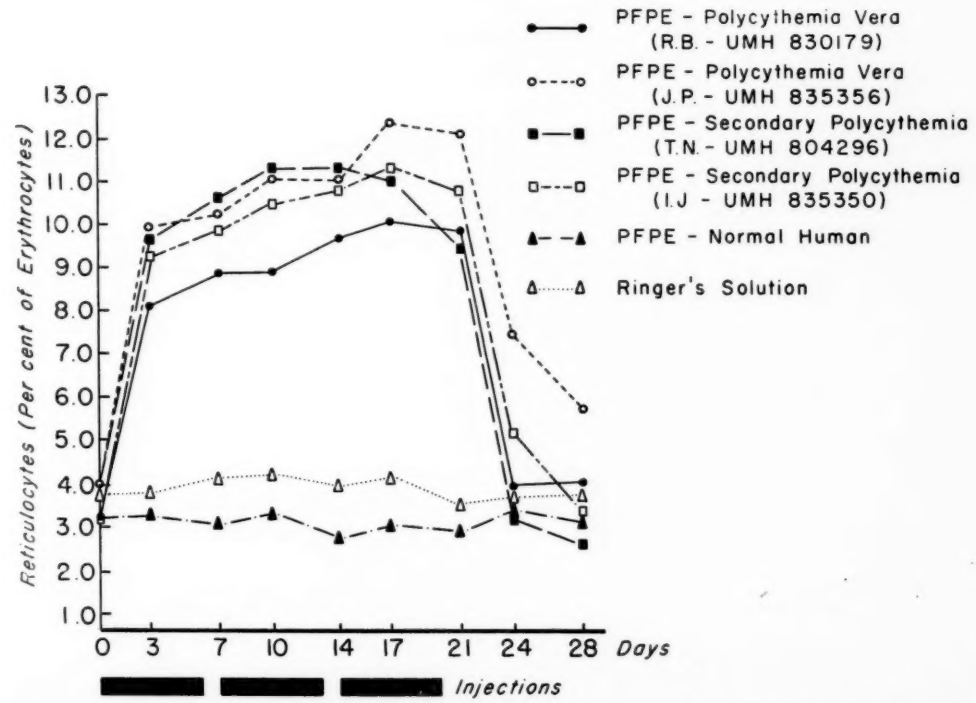

Fig. 2.—Reticulocytosis in rats injected with plasma extracts from patients with polycythemia vera and secondary polycythemia. Mean of reticulocyte counts of rats receiving the above described materials.

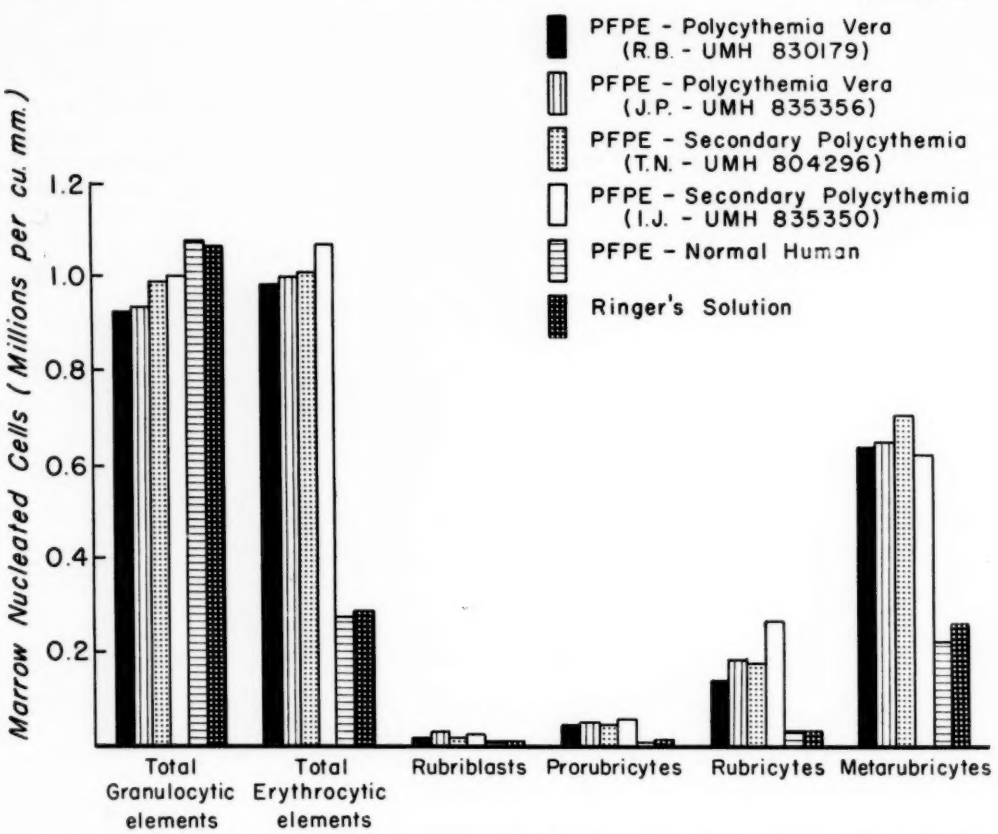

Fig. 3.—Marrow nucleated cell counts demonstrating erythrocytic hyperplasia involving all red cell precursors in animals receiving human "polycythemic" plasma extracts. Mean cell counts of 4 rats in each group given human "polycythemic" plasma extracts, 8 rats injected with normal human plasma extracts, and 14 from the control group.

erythrocytosis and reticulocytosis continued to increase throughout the injection period, the greatest increase over the baseline values occurred during the first week with the reticulocytes showing a definite tendency to level off as the injections were continued. There was a prompt return to normal values after the injections were stopped. As previously noted,[10] the erythrocytosis was due to an increase in small cells without appreciable elevation in the hemoglobin or hematocrit (Tables III and IV). The mean corpuscular hemoglobin concentra-

TABLE III. AVERAGE HEMOGLOBIN DETERMINATIONS (GRAMS PER CENT)

GROUPS	BASELINE	1 WEEK	2 WEEKS	3 WEEKS*	4 WEEKS
PFPE—normal human	14.2	14.2	14.3	15.4	14.5
Ringer's solution	14.2	13.7	13.9	14.7	14.8
PFPE—polycythemia vera (R. B.—UMH 830179)	14.5	14.3	14.7	15.5	13.9
PFPE—polycythemia vera (J. P.—UMH 835356)	14.5	14.1	14.3	14.8	14.7
PFPE—secondary polycythemia (T. N.—UMH 804296)	13.9	13.9	14.6	15.9	14.2
PFPE—secondary polycythemia (I. J.—UMH 835350)	14.0	14.4	13.7	15.1	15.2

*Injections discontinued.

tion remained unchanged (Table V) while the mean corpuscular volume (Table VI) and the mean corpuscular hemoglobin (Table VII) fell in the animals showing the peripheral erythrocytosis. The small size of the erythrocytes was evident in both the hemacytometer and on the stained films. There was no evidence of accelerated erythropoiesis in the rats injected with normal human PFPE and their erythroid values and marrow findings closely paralleled those of the control group given Ringer's solution.

TABLE IV. AVERAGE HEMATOCRIT LEVELS (VOLUMES PER CENT)

GROUPS	BASELINE	1 WEEK	2 WEEKS	3 WEEKS*	4 WEEKS
PFPE—normal human	44.2	45.3	43.9	44.7	44.4
Ringer's solution	44.3	44.5	43.9	44.0	44.1
PFPE—polycythemia vera (R. B.—UMH 830179)	44.6	46.2	45.3	45.5	43.4
PFPE—polycythemia vera (J. P.—UMH 835356)	44.8	44.8	44.8	45.1	46.3
PFPE—secondary polycythemia (T. N.—UMH 804296)	42.8	43.9	44.0	46.3	44.0
PFPE—secondary polycythemia I. J.—UMH 835350)	43.0	44.8	42.8	43.4	45.1

*Injections discontinued.

TABLE V. AVERAGE MEAN CORPUSCULAR HEMOGLOBIN CONCENTRATION (PER CENT)

GROUPS	BASELINE	1 WEEK	2 WEEKS	3 WEEKS*	4 WEEKS
PFPE—normal human	32.1	31.3	32.5	34.4	32.6
Ringer's solution	32.0	30.7	31.6	33.4	33.5
PFPE—polycythemia vera (R. B.—UMH 830179)	32.5	30.9	32.4	34.0	32.0
PFPE—polycythemia vera (J. P.—UMH 835356)	32.3	31.4	31.9	32.8	31.7
PFPE—secondary polycythemia (T. N.—UMH 804296)	32.4	31.6	33.1	34.3	32.2
PFPE—secondary polycythemia (I. J.—UMH 835350)	32.5	32.1	32.0	34.7	33.7

*Injections discontinued.

TABLE VI. AVERAGE MEAN CORPUSCULAR VOLUME (CUBIC MICRONS)

GROUPS	BASELINE	1 WEEK	2 WEEKS	3 WEEKS*	4 WEEKS
PFPE—normal human	53.7	53.4	52.5	53.9	48.9
Ringer's solution	53.3	55.5	55.0	54.5	51.8
PFPE—polycythemia vera (R. B.—UMH 830179)	53.8	43.3	39.9	36.8	49.1
PFPE—polycythemia vera (J. P.—UMH 835356)	53.5	40.5	37.0	36.1	53.7
PFPE—secondary polycythemia (T. N.—UMH 804296)	57.9	41.4	36.3	36.0	52.8
PFPE—secondary polycythemia (I. J.—UMH 835350)	50.6	38.1	33.9	54.3	33.6

*Injections discontinued.

TABLE VII. AVERAGE MEAN CORPUSCULAR HEMOGLOBIN (MICROMICROGRAMS)

GROUPS	BASELINE	1 WEEK	2 WEEKS	3 WEEKS*	4 WEEKS
PFPE—normal human	17.2	16.7	17.1	18.5	15.9
Ringer's solution	17.1	17.1	17.4	18.2	17.4
PFPE—polycythemia vera (R. B.—UMH 830179)	17.5	13.4	12.9	12.9	15.7
PFPE—polycythemia vera (J. P.—UMH 835356)	17.3	12.7	11.8	11.8	17.1
PFPE—secondary polycythemia (T. N.—UMH 804296)	18.8	13.1	12.0	12.3	17.0
PFPE—secondary polycythemia (I. J.—UMH 835350)	16.4	12.2	10.8	11.7	18.3

*Injections discontinued.

The increase in total marrow cellularity in the rats exhibiting the erythrocytosis and reticulocytosis was due solely to a three- to fourfold increase in erythrocytic precursors involving a roughly proportional increase in all forms, as shown in Fig. 3 and Table II. The relative constancy of the granulocytic elements is evident.

All animals remained healthy throughout the experimental period and exhibited no adverse local or generalized effects to the injections. The rats receiving the human plasma extracts gained weight in amounts comparable to the animals injected with Ringer's solution.

DISCUSSION

The demonstration of an erythropoietic-stimulating factor in human "polycythemic" plasma by the bioassay method herein employed may provoke a number of speculations. It confirms the existence of a humoral erythropoietic factor in man as previously postulated and supported by such observations as those of Stohlman and associates[12] and Schmid and Gilbertsen[13] in patients with regional hypoxia. It has been shown that anemia per se is not a requirement for the production of the plasma erythropoietic-stimulating factor in experiments such as those of Reissmann,[14] who utilized parabiotic rats with hypoxia as the stimulus to its formation. The presence of this factor in the plasma of patients with polycythemia vera in the absence of anemia or reduced oxygen tension is additional evidence that the humoral factor may exert important physiologic and pathophysiologic regulatory control over erythropoiesis.

Decreased arterial oxygen saturation in Patients T. N. and I. J. was presumably the stimulus to the overproduction of the plasma erythropoietic-stimulating factor, which in turn evoked the accelerated erythropoiesis and the subsequent elevation in the peripheral erythroid values. This would explain the secondary erythrocytosis seen in patients with hypoxia, whether due to circulatory defects, pulmonary disease, or lowered atmospheric oxygen tension. In anemic states, although the arterial oxygen saturation may be normal, the oxygen carrying capacity of the blood is reduced. In view of the demonstrated effect of hypoxia upon the formation of the plasma erythropoietic-stimulating

factor, anemic anoxia would seem to be the stimulus to its production in response to anemia. Erslev[15] has shown that in instances of dilution anemia there is no increase in erythropoietic activity despite the reduced oxygen content of arterial blood. He concludes that the stimulus to erythropoietic function must depend on the tissue tension of oxygen rather than the oxygen content of arterial blood. Tissue oxygen tension, therefore, plays an important but most likely an indirect role in erythropoiesis. It has been shown that the bone marrow oxygen tension in acute and chronic anemia is normal,[16] a finding which is in agreement with the intermediary role of hypoxia in erythropoietic control rather than a direct action on hemopoietic elements. In addition, studies of bone marrow in vitro have shown that a reduction in oxygen tension depresses rather than stimulates erythropoietic activity.[17, 18] Since hypoxia appears to exert its effect on erythropoiesis through the humoral-stimulating factor, the fact that high tensions of inspired oxygen depress erythrocytogenesis in the human subject[19] provides additional evidence for the possible role of the humoral factor in the maintenance of normal erythroid values.

The site of production of the plasma erythropoietic-stimulating factor is not known. Erslev[15] has suggested that it is formed in some relatively nonvital cellular system or organ other than bone marrow, thus explaining its presence in anemic or hypoxic states where cardiac and pulmonary adjustments maintain the oxygen supply to such vital organs as the brain. Recent studies by Erslev and Lavietes[20] and Linman and Bethell[21] have shown, respectively, that nitrogen mustard or x-irradiation administered prior to rendering rabbits anemic does not prevent the formation of the factor. These observations add support to the theory that the site of production of the humoral erythropoietic-stimulating factor is other than myeloid tissue. Rabbits made anemic by total body irradiation alone also produce the factor,[21] thus indicating that a regenerative marrow is not a prerequisite to its formation and the factor is not a product of hyperactive myeloid elements.

The presence in polycythemia vera of increased amounts of an erythropoietic-stimulating factor raises several interesting hypotheses. Since polycythemia vera was initially described by Vaquez[22] in 1892 and clearly established as a specific disease entity by Osler[23] in 1903, its etiology and pathogenesis have been subject to speculation. The more commonly held theories of causation include hypoxia or reduced oxygen tension, an abnormality of the hypophyseal-hypothalamic system, a neoplastic process, and a derangement in the physiologic regulatory mechanism responsible for maintaining the normal erythroid steady state. In view of the known stimulus to erythropoiesis induced by anoxia, its possible role in the etiology of polycythemia vera has been especially stressed. There is, however, no evidence of an oxygen deficit in polycythemia vera. The arterial[11] and sternal marrow blood[24, 25] oxygen saturation in patients with polycythemia vera is normal. This is in contrast to the reduction in both arterial and marrow oxygen saturation in patients with secondary polycythemia. A possible defect in gas transfer in the pulmonary alveoli has been suggested,[26] but not proved.[27] It has been shown by Lawrence and his associates[28] that the inhalation of 50 per cent oxygen depresses plasma iron turnover and red cell

iron uptake in secondary polycythemia, but not in polycythemia vera, thus affording further evidence that the etiology of the latter condition is not due to hypoxia, at least in the ordinary sense. The possibility is not excluded of vascular bone marrow lesions, as described by Reznikoff, Foot, and Bethea[29] in 1935, producing localized hypoxia. However, the platelet and granulocyte changes commonly associated with polycythemia vera indicate a different mechanism than simple hypoxia.

Polycythemia has been observed in association with lesions in and around the pituitary and the midbrain,[30-33] but abnormalities of the hypophyseal-hypothalamic system in patients presenting the characteristic changes of polycythemia vera have not been demonstrated. Although there is evidence suggesting a regulatory function of the midbrain on erythropoiesis,[34] it has not been possible to implicate this area in the pathogenesis of polycythemia vera. It has been shown recently that the anterior pituitary is intimately associated with erythropoiesis.[35] Hypophysectomy produces an anemia in the experimental animal which can be corrected, and in fact polycythemia produced,[36] by the administration of a sheep anterior pituitary preparation. This erythropoietically active material is distinct from the other known pituitary hormones[37] and does not require the presence of target organs such as the adrenals, thyroid, or gonads to exert its effect upon erythropoiesis.[38] Hypophysectomy, while not preventing, does impair the normal erythropoietic response to hypoxia.[38] The anterior pituitary factor has also been shown to prevent neonatal anemia in the newborn rat[39] and will produce erythropoietic stimulation in the adult intact rat. The anemia accompanying hypophysectomy can be corrected by the administration of cobalt,[40] thus indicating that the polycythemia-producing action of cobalt is independent of the pituitary gland. Further investigation is required before the pituitary can be assigned a specific role in normal and abnormal erythropoiesis. The possibility that the plasma erythropoietic-stimulating factor and the anterior pituitary factor are identical must be considered. The fact that hypophysectomy does not prevent erythropoietic stimulation secondary to hypoxia is against such identity. Although hypophysectomized animals do not show the same degree of response to reduced oxygen tension as normal animals, they do demonstrate accelerated erythropoiesis. Van Dyke and associates[38] have reported that hypophysectomized rats put in a chamber at 22,000 feet daily for 14 days, 6 hours a day, showed a 40 per cent increase in circulating red cell volume over that of hypophysectomized controls maintained at sea level. When they were exposed to the same simulated altitude for 27 days, a 57 per cent increase occurred, bringing the red cell volume back to normal levels. These animals, therefore, while not developing polycythemic values as do normal animals, did show an erythropoietic response to lowered oxygen tension. The difference may be explained by their inability to respond beyond a certain level because of other hemopoietic changes induced by removal of the pituitary, rather than by the assumption that the anterior pituitary is the chief regulatory center of erythropoiesis with the hypoxic stimulus being mediated through this organ. Furthermore, the failure to demonstrate activity

in rats of a beef pituitary[35] erythropoietic factor is incompatible with the absence of species specificity noted for the plasma erythropoietic-stimulating factor.

The neoplastic concept of polycythemia vera, which is most widely held, is supported by its well-documented association with leukemia and related myeloproliferative disorders.

There remains the theory that some derangement occurs in polycythemia vera affecting the physiologic regulation of erythropoiesis. In view of the meager knowledge concerning the control of erythrocyte production, it is readily apparent why this hypothesis has not received acceptance. The demonstration of increased amounts of an erythropoietic-stimulating factor in the plasma of patients with polycythemia vera supports the possibility that a disturbance of erythropoietic regulation may be of pathogenetic importance.

The existence of a plasma erythropoietic-stimulating factor in patients with secondary polycythemia might be anticipated in view of the known effect of hypoxia. Its presence in patients with polycythemia vera, however, is not so readily explained. Unless the factor was produced by active myeloid elements, considerable significance must be attributed to its demonstration in patients with polycythemia vera. The observations previously discussed concerning the lack of effect of nitrogen mustard[20] or x-irradiation[21] on production of the factor would seem to exclude hemopoietic tissue as the site of its formation or a regenerative marrow as a prerequisite for its elaboration. The phenomenon would, therefore, appear to be of possible primary importance in the pathogenesis of polycythemia vera and not merely a manifestation of heightened erythropoietic activity. The assay of plasma from radiation-treated patients with polycythemia vera with normal erythroid values is planned. The demonstration of the plasma erythropoietic factor in such patients would support its etiologic role in the disease, although a negative result would not exclude such a possibility. The accelerated granulopoiesis and thrombopoiesis commonly associated with polycythemia vera but not with secondary polycythemia have also to be explained. It has been shown that "anemic" plasma does not produce such changes in the recipient animal.[4, 9] Blood leukocyte and platelet observations were not made in the study being reported, but the constancy of the marrow granulocytic numbers is evidence against any effect on these hemopoietic elements exerted by the plasma factor. The question deserves further study, and the possible influence of other humoral factors on granulocyte and platelet formation should receive consideration.

In respect to the possible mode of action of the erythropoietic-stimulating factor, it has been suggested by marrow studies,[10] although the dynamics have not been observed, that in the nonanemic rat the factor promotes increased cellular division of already existent erythrocytic precursors rather than augmented diversion of multipotential reticulum cells into erythrocyte production. The erythrocytes resulting from accelerated cellular division are small and there is no appreciable elevation in the circulating hemoglobin or red cell mass. Evidence has also been obtained indicating that these microcytes have a decreased lifespan, thus explaining, in part, the rapid return to normal values

following cessation of the administration of the plasma factor. An analogous situation with respect to erythrocyte survival in polycythemia is suggested by the observations of Berlin and his co-workers,[41] using the C^{14}-methyl-labeled glycine technique, that the red cells of patients with polycythemia vera can be divided into two classes. One has a normal lifespan and the second class a survival time of only a few days. It is their conclusion that it is the rapid turnover of these short-lived cells that is largely responsible for the greatly increased rate of utilization of iron in polycythemia vera. Huff and co-workers[42] have shown that the red cell iron turnover in patients with polycythemia vera is approximately five times normal and about twice normal in secondary polycythemia. Microcytes have been noted in the blood of patients with polycythemia vera,[43] as has evidence of increased blood destruction as suggested by bilirubinemia and increased urobilinogen in the urine and feces.[44] Merino,[45] in his study of the erythropoietic stimulation induced by moving normal male subjects from sea level to an altitude of 14,900 feet, reported a lack of parallelism between the increase in red cell count and the hemoglobin and hematocrit with a corresponding degree of microcytosis during the first few days after their arrival. All of these subjects showed a moderate increase in serum bilirubin in the indirect form and, with a single exception, the fecal urobilinogen increased but in only one instance did it exceed the upper limits of normal.

It is treacherous to theorize concerning the mode of action of the plasma erythropoietic-stimulating factor in anemic states from observations on the normal animal. Some observors have noted increases in the hemoglobin and/or hematocrit values of rabbits[4] and rats[8, 9] following the administration of the plasma erythropoietic factor, and we have observed such changes in occasional rats. In monkeys, Erslev and associates[5] reported reticulocytosis without other change in peripheral erythroid values following the administration of "anemic" serum. Our failure to obtain consistent and significant elevations of hemoglobin and hematocrit levels in normal rats given plasma extracts from polycythemic patients with striking increases in these erythroid determinations may be due in part to species differences and in part to conditions of the experiment, especially temporal factors. The duration of stimulation would seem to be of paramount importance in determining the erythropoietic response, since a three-week experimental period is hardly comparable to the chronic stimulation present in patients with both primary and secondary polycythemia.

Although normal human plasma did not produce increased erythropoietic activity in the recipient rat, it need not thereby be inferred that the erythrocyte stimulatory factor is absent in the normal state or active only in pathologic situations. Differences in concentration may explain the presence or absence of response in the normal recipient animal.

The biochemical nature of the plasma erythropoietic-stimulating factor is not known. It is stable over a wide range of temperature, acid-soluble, and is not precipitated by perchloric acid. The absence of activity in ashed plasma extracts establishes it as an organic compound. Ultraviolet absorption studies

have shown that boiling and precipitation with perchloric acid removed 92.3 and 94.1 per cent of the materials absorbed at wave lengths of 260 and 28 mμ, respectively. Since the removal of such substances does not alter the erythropoietic activity of the plasma, this observation is strongly indicative of the nonprotein nature of the factor.

Preliminary studies in this laboratory have shown that the plasma erythropoietic factor is not removed by passage through either Dowex-50-X8, NH_4^+, or Dowex-1-X10, $COOH^-$ ion exchange resin columns. It is also ether-soluble at neutral pH. Such observations, if confirmed, will exclude a large number of biologically active compounds.

In 1949, Sandler[46] reported investigations of the erythropoietic stimulatory effect of yellow bone marrow extracts. He was able to isolate batyl alcohol or the monoglycerol ether of n-octadecyl alcohol [$CH_3(CH_2)_{17}$-O-CH_2-CH(OH)-CH_2(OH)] from the yellow bone marrow of cattle and showed that this material was capable of stimulating erythropoiesis in normal and benzene-treated rats. An optically inactive synthetic form was given orally to normal human subjects and evoked a reticulocyte response. It is of interest that the preliminary biochemical observations described above are compatible with a compound such as α-n-octadecyl glyceryl ether.

Assay in the rat of human nonprotein plasma extracts from a variety of disorders associated with impaired erythropoiesis should yield important information on the mechanisms governing erythropoiesis and their derangements.

SUMMARY

1. Human nonprotein plasma extracts from patients with polycythemia vera and secondary polycythemia are capable of stimulating erythropoiesis in the normal rat, thus affording direct evidence for the existence of a humoral erythropoietic factor in man.

2. In the recipient rat this erythropoietic stimulation is manifested by erythrocytosis, reticulocytosis, and increased marrow erythropoietic activity without an appreciable increase in hemoglobin or hematocrit levels. It is suggested that species differences and the duration of erythropoietic stimulation may determine increase in red cell mass.

3. It is postulated that in the rat over a short experimental period, the plasma erythropoietic-stimulating factor increases the number of cellular divisions of already existent erythrocyte precursors yielding microcytes with a shortened survival time. Such a concept receives support from previous observations that some of the erythrocytes in polycythemia vera and secondary polycythemia are abnormal.

4. Hypoxia is believed to be the stimulus to the formation of the humoral factor in patients with secondary polycythemia.

5. In view of the absence of demonstrable hypoxia in patients with polycythemia vera and the experimental evidence against the production of the

factor by hemopoietic tissue, it is suggested that the plasma erythropoietic-stimulating factor may have etiologic significance in polycythemia vera in that its increased amount or augmented activity may be indicative of a derangement of the normal physiologic mechanisms governing erythropoiesis.

6. Although normal human plasma did not possess erythropoietic activity by the bioassay method used, the absence of response may be due to its low concentration in normal plasma rather than to its absence.

7. Further investigations involving the assay in the rat of human non-protein plasma extracts from a variety of hematologic disorders should add important information to our present knowledge of the regulatory mechanisms controlling both normal and abnormal erythropoiesis.

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LABORATORY METHODS

A METHOD FOR RADIOIODINATION OF ANTIBODY PROTEIN

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INTRODUCTION

RADIOACTIVE antitumor antibodies, of high specific activity, have potentialities in the localization and therapy of certain tumors. Several methods for radioiodination of antisera and gamma globulins from such sera have been published.[1-5] For the most part, these methods are suitable for trace labeling of antibodies to be used in studies of the antigen-antibody reaction, or for localization studies in in vivo experiments. However, when antibody protein is to be labeled to a higher range of specific activity, these methods have several drawbacks. Probably the best method in this respect, that of Gilmore,[4] results in fairly high specific activity albumin preparations but in our experience tends to denature gamma globulin, at least to a point where antibody titer is markedly diminished.

This report gives the details of a method which we have found satisfactory for the preparation of antibody protein labeled to specific activities in the range of 50 to 70 μc per milligram, without loss of specificity or antibody titer. No special apparatus is required.

MATERIALS AND METHODS

The following procedures were carried out at temperatures between 0° and 5° C.

Twenty-five hundredths milliliter 0.019 per cent (w/v) NaI solution, the required amount of I^{131} radioactivity (NaI^{131} carrier-free) and 0.25 ml. 0.006 per cent (w/v) $NaIO_3$ solution, was placed in a small beaker. Five-tenths milliliter glacial acetic acid, followed by 2.5 ml. cold water, was added and the mixture allowed to stand 15 minutes, after which the pH was adjusted to 6 to 6.5 with 1.0 N sodium hydroxide. Ten milligrams of gamma globulin dissolved in sufficient pH 8.5 phosphate buffer to bring the total volume to 10 ml. (or other convenient volume) was cooled in a separate container and the radioactive mixture added. The mixture was allowed to stand four hours, after which it was dialyzed against 0.9 per cent sodium chloride for 12 to 18 hours. (Removal of free radioiodide can also be accomplished by the use of ion-exchange resins, such as Dowex 2 anionic resin.)

Radioactivity was determined by counting 2 ml. aliquots of suitable dilutions of the dialyzed material in a well-type scintillation counter.

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RESULTS

With use of the above procedure, radioactive gamma globulin solutions of specific activity up to 70 μc per milligram have been prepared. The protein, prepared from antisera to *Proteus vulgaris* OX19, by the method of Cohn and Deutsch,[6] was titered against this organism with use of the macroscopic tube agglutination technique. The titer before radioiodination was 1:1,560 and remained unchanged after labeling. When the gamma globulin was titered against several related strains of *Proteus vulgaris,* before radioiodination, titers of less than 1:10 were obtained. No increase in these titers was noted after radioiodine treatment, indicating no change in specificity, at least as observable by the tests used.

DISCUSSION

The principle of the method involves the quantitative liberation of iodine according to the reaction:

$$5\ NaI + NaIO_3 + 6\ CH_3COOH = 3\ I_2 + 6\ Na.OOC.CH_3 + 3\ H_2O$$

A procedure based on a similar principle was suggested by Francis[3] but was performed in the presence of excess NaI, thus making unavailable about one-half the added radioactivity. Since only 25 to 30 per cent of the available radioiodine is taken up by the protein, this constitutes a definite drawback when a product of high specific activity is desired. These workers also used the reaction without excess NaI, followed by ether extraction of liberated iodine, but did not recommend this procedure for the iodination of proteins.

Essentially, the present method involves the exchange of a minute amount of carrier iodide with radioactive iodide, and the liberation of the labeled iodine. Since this reaction requires an acid pH, the solution must subsequently be adjusted to a pH as close to neutrality as is possible without recombination of the liberated iodine with sodium ions. The actual reaction with protein should be performed at a basic pH to minimize the oxidizing action of iodine; hence the use of basic buffer. Gamma globulin has been used instead of antiserum, since combination of iodine to varying extents with other serum proteins is excluded, thus yielding a product of known specific activity. It is essential to use protein preparations with good solubility and other desirable physical properties, since poor quality preparations are likely to denature and to affect reproducibility of results.

Francis[3] has shown that the precipitating power of antibody is markedly reduced if more than 6.5 molecules of iodine are introduced per molecule of antibody. It is also recognized that the body may dispose of protein substituted above this limiting amount at a fractional rate different from that observed with the untreated material. The present method does not involve the substitution of more than 2 to 3 molecules of iodine per molecule of gamma globulin, which is in accord with the absence of change in antibody titer.

Recently Berson[7] has noted, in I^{131}-labeled insulin, certain changes which appear to be the result of radiation damage. Antibody protein labeled as high as 70 μc per milligram may therefore have suffered such damage in ways too subtle to be noted by the criteria applied.

SUMMARY

A simple method for radioiodination of antibody protein (gamma globulin fractions from immune sera), yielding preparations of specific activity up to 70 μc per milligram, is described. Antibody titer and specificity are unaffected. The possibility of radiation damage is not excluded.

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DRUG-SUSCEPTIBILITY TESTS FOR MYCOBACTERIUM TUBERCULOSIS

A Comparative Study of Standard and Semiquantitative Techniques

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INTRODUCTION

VARIATION in results of repeated drug-susceptibility tests and frequent lack of clinical correlation have been widely criticized and have led to a certain lack of confidence by clinicians. It has been shown by several investigators[1-7] that this may be a result, in part, of the relatively large inoculum used with masking of the true proportions of susceptible and resistant cells. Therefore, an attempt was made to devise a *semiquantitative* test, sufficiently simple for routine use, that would give a reasonable estimate of the proportion of susceptible and resistant tubercle bacilli. We have proceeded with the thought that such a test, used less frequently, and perhaps in selected cases, would provide more dependable information and reduce the total work by the laboratory in this sphere.

MATERIALS AND METHODS

Patients' sputa which yielded consistently heavy growth, resistant to one or more drugs, were obtained for direct tests. Twenty-four-hour specimens were digested and concentrated according to the NaOH technique.[8] The neutralized sediment was washed once in 20 ml. of 0.1 per cent bovine albumin solution. Three milliliters of 0.1 per cent bovine albumin solution was added to the sputum concentrate to obtain sufficient volume for the performing of comparative tests.

Subcultures of drug-resistant bacilli isolated from patients by the clinical laboratory were used for the indirect tests. A suspension in 0.1 per cent bovine albumin solution was prepared from the entire growth by means of a glass grinder. Heavy particles were removed by low speed centrifugation. The density of the suspension was adjusted in a Klett-Summerson photoelectric colorimeter to the equivalent of a McFarland No. 1 standard.

The *standard* method for drug-susceptibility tests was carried out essentially as recommended by the Subcommittee on Laboratory Methods.[8]

Sputum concentrate and bacillary suspension were diluted to yield countable colonies (25 to 250) in the *semiquantitative* test. Sputum concentrate was diluted 1:10, 1:100, and 1:1,000 when the smear showed 1 to 25 acid-fast bacilli per oil immersion field. Dilutions of 1:100, 1:1,000, and 1:10,000 were made if the smear showed over 25 bacilli per oil immersion field. Each bacillary suspension was diluted 1:1,000, 1:10,000, and 1:100,000.

In all tests, 0.1 ml. of undiluted and diluted sputum concentrates and bacillary suspensions was inoculated on duplicate drug-free and drug-containing solid egg media. Egg yolk agar medium (agar-agar, 2 per cent; egg yolk, 25 per cent; egg white, 18 per cent;

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malachite green, 0.03 per cent) was used in the beginning of the study for streptomycin susceptibility tests. Modified Lowenstein-Jensen medium[8] was used for streptomycin, p-aminosalicyclic acid, isoniazid, and viomycin susceptibility tests.

All tests were read after 3, 4, and 6 weeks' incubation at 37° C.

RESULTS

The results of 30 direct and 20 indirect drug-susceptibility tests of bacilli from 28 patients performed by *standard* and *semiquantitative* methods are summarized in Tables I and II. Reasonable agreement (within 30 per cent) between

TABLE I. RESULTS OF 30 DIRECT DRUG-SUSCEPTIBILITY TESTS BY STANDARD AND SEMIQUANTITATIVE TECHNIQUES

	STANDARD METHOD		SEMIQUANTITATIVE METHOD		
PATIENT	READINGS*	APPARENT PER CENT RESISTANT CELLS†	DILUTION	AVERAGE COLONY COUNTS*	APPROXIMATE PER CENT RESISTANT CELLS†
O. S.	SM 4+, 4+, 4+	100	1:1,000	SM 122, 14, 2	11
	INH 4+, 4+, 4+	100		INH 130, 133, 128	100
F. R.	SM 4+, 4+, (34)	100	1:1,000	SM 26, 28, 0	100
	PAS 4+, 2+, 0	50		PAS 53, 0, 0	0
	INH 4+, 4+, 0	0		INH 53, 9, 0	0
F. C.	SM 4+, 4+, 4+	100	1:1,000	SM 212, 191, 105	90
	PAS 4+, 4+, 0	100		PAS 179, 90, 0	50
	INH 4+, 4+, 4+	100		INH 179, 170, 176	100
F. D.	SM 4+, 2+, 0	50	1:100	SM 111, 72, 0	65
	PAS 4+, 2+, 0	50	1:1,000	PAS 28, 4, 0	15
	INH 4+, 4+, 4+	100		INH 28, 25, 20	70
M. O.	SM 4+, 4+, 4+	100	1:1,000	SM 41, 41, 41	100
	PAS 4+, 4+, 0	100		PAS 198, 14, 0	7
	INH 4+, 4+, 4+	100		INH 198, 152, 69	35
A. S.	PAS 4+, 2+, 0	50	1:1,000	PAS 173, 0, 0	0
	INH 4+, 4+, 4+	100		INH 173, 158, 29	27
K. S.	SM 4+, 4+, 4+	100	1:1,000	SM 164, 132, 119	80
J. O.	SM 4+, 4+, (48)	100	1:10,000	SM 31, 11, 0	35
	PAS 4+, 3+, 0	75		PAS 37, 8, 0	21
	INH 4+, 4+, 2+	50		INH 37, 7, 0	0
H. M.	SM 4+, 3+, 3+	75	1:100	SM 81, 56, 0	69
	INH 4+, 4+, 3+	75		INH 81, 88, 28	35
E. G.	SM 4+, 4+, 4+	100	1:1,000	SM 71, 0, 0	0
	INH 4+, 4+, 4+	100		INH 71, 37, 16	23
J. B.	SM 4+, 4+, 4+	100	1:1,000	SM 34, 2, 1	6
	INH 4+, 4+, 4+	100		INH 34, 35, 2	6
R. M.	INH 4+, 4+, 3+	75	1:10	INH 241, 235, 173	72
H. V.	PAS 4+, 4+, 0	100	1:10,000	PAS 45, 14, 0	31
J. H.	INH 4+, 4+, 4+	100	1:100	INH 145, 170, 126	87
W. F.	SM 4+, 4+, 0	100	1:1,000	SM 27, 0, 0	0

*Drug concentration in mcg./ml. of medium:
SM 0, 10, 100 (egg yolk-agar medium)
PAS 0, 10, 100 (modified Lowenstein-Jensen medium)
INH 0, 1, 5

4+ Surface of medium entirely covered with confluent growth.
3+ Growth heavy, but not confluent.
2+ Innumerable distinct colonies.
1+ 50–200 colonies.
( ) Actual colony count if less than 50.

†Based on amount of growth in tube containing 10 mcg. SM/ml., 10 mcg. PAS/ml. or 5 mcg. INH/ml. as compared to growth in control tube.

the two methods was obtained in 11 of 30 direct and 8 of 20 indirect tests. In the remaining comparisons the *semiquantitative* procedure always showed a considerably lower proportion of resistant and a higher proportion of susceptible cells than the *standard* method. In several tests (5 direct and 4 indirect) where strong resistance was indicated by the latter, none were found with the former technique.

TABLE II. RESULTS OF 20 INDIRECT DRUG-SUSCEPTIBILITY TESTS BY STANDARD AND SEMIQUANTITATIVE METHODS

	STANDARD METHOD		SEMIQUANTITATIVE METHOD		
PATIENT	READINGS*	APPARENT PER CENT RESISTANT CELLS†	DILUTION	AVERAGE COLONY COUNTS*	APPROXIMATE PER CENT RESISTANT CELLS†
J. S.	INH 4+, 4+, 4+, 4+	100	1:1,000	INH 238, 225, 222, 110	93
R. M.	INH 4+, 4+, 4+, 4+	100	1:10,000	INH 27, 27, 23, 13	85
J. M.	INH 4+, 4+, 4+, 0	100	1:10,000	INH 191, 190, 199, 0	100
E. B.	SM 4+, 3+, (1)	75	1:10,000	SM 25, 4, 0	16
	INH 4+, 4+, 4+, 4+	100		INH 25, 24, 20, 21	84
R. W.	VM 4+, 2+, 0	50	1:10,000	VM 67, 0, 0	0
H. M.	SM 4+, 4+, 2+	100	1:1,000	SM 243, 217, 0	89
	INH 4+, 4+, 3+, 1+	75		INH 243, 236, 9, 0	4
O. G.	SM 4+, 4+, 2+	100	1:10,000	SM 66, 18, 0	27
R. N.	SM 4+, 2+, (11)	50	1:10,000	SM 97, 0, 0	0
	INH 4+, 4+, 2+, 2+	50		INH 97, 1, 0, 0	0
R. A.	INH 4+, 4+, 4+, 4+	100	1:1,000	INH 93, 41, 42, 13	45
S. G.	SM 4+, 4+, 4+	100	1:100,000	SM 53, 44, 47	83
C. M.	INH 4+, 4+, 2+, 0	50	1:100,000	INH 34, 1, 0, 0	0
J. V.	INH 4+, 4+, 4+, 2+	100	1:100,000	INH 127, 28, 2, 0	2
O. S.	SM 4+, 2+, 0	50	1:10,000	SM 81, 0, 0	0
	INH 4+, 4+, 4+, 4+	100		INH 81, 84, 87, 15	100
J. C.	INH 4+, 4+, 4+, 0	100	1:10,000	INH 91, 92, 6, 0	7
T. M.	SM 4+, 4+, 4+, ‡	100	1:100,000	SM 22, 16, 9, ‡	73
	INH 4+, 4+, 4+, 4+	100		INH 28, 16, 16, 16	57

*Drug concentrations in mcg./ml. of modified Lowenstein-Jensen medium:

SM 0, 10, 100
PAS 0, 10, 100
INH 0, 1, 5, 25
VM 0, 10, 100

†Based on amount of growth in tube containing 10 mcg. SM/ml., 5 mcg. INH/ml., 10 mcg. PAS/ml. or 10 mcg. VM/ml. as compared to growth in control tube.

‡Egg yolk-agar medium used.

DISCUSSION

The *standard* method is more sensitive for resistant cells which may be of real clinical significance. This was suggested by Mitchison[9] who at the same time felt that a still more reliable test was needed. For indirect tests, Diagnostic Standards[10] recommends sufficient dilution of inoculum for prevention of masking of the presence of susceptible cells. Bogen[11] suggests a compromise inoculum

of 10,000 bacilli. Possibly the presumed advantages of both tests could be retained by use of a heavy inoculum for one or more tests of tubes and suitable dilutions in other sets as was done for our comparisons. This could be done in selected cases but may be too elaborate for routine purposes.

The clinical value of a *semiquantitative* test in which small inocula are employed remains to be seen.

SUMMARY

A comparison of a *standard* with a *semiquantitative* method for testing the drug-susceptibility of *Mycobacterium tuberculosis* showed reasonable agreement in 11 of 30 direct and 8 of 20 indirect tests. Disagreement in the results of the remaining 19 direct and 12 indirect tests was due to differences in inoculum. The *standard* method is more sensitive for resistant cells, but their proportions are uncertain. The *semiquantitative* procedure gives a more balanced picture of the ratio of susceptible and resistant bacilli, although it is less sensitive for either because of the smaller inocula. Its lower sensitivity can be enhanced by inclusion of a lower dilution inoculum.

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A VERSATILE PAPER ELECTROPHORESIS APPARATUS

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INTRODUCTION

A STUDY of radiation effects on beef serum albumin required a paper electrophoresis apparatus with the following specifications: (1) facilities for using both paper strips and sheets of commercially available dimensions; (2) free-hanging paper; (3) provision for indirect cooling of the paper and of the electrodes; (4) maintenance of a constant pH in the electrodes; (5) elimination of multiple electrode compartments; (6) provisions for continuously applying samples to sheets of electrophoresis paper at controlled rates; (7) controlled flow of the buffer curtain in two-dimensional experiments; (8) arrangements permitting the concurrent analysis of two samples; (9) facilities for simple exchange of electrophoresis paper; (10) continuously variable D.C., power for the range from 100 to 1,250 V. with a total operating load of 60 Ma. from two outlets, each with a voltage regulation of ±1 per cent.

MATERIALS, METHODS, AND RESULTS

The apparatus is illustrated diagrammatically in Figs. 1 to 3. It consists essentially of two pairs of mobile flow-electrodes which can be placed either in the horizontal or vertical position to accommodate vertical strips of filter paper or two sheets up to 18 by 22 inches. The electrodes are of the continuously flowing buffer type,[1, 3-6] which eliminates the usual conductivity bridges and transfer tanks of electrode reservoirs. The electrodes are mounted on Lucite supports and consist of platinum wire within a woven nylon tube[2] which serves as a support for a Visking cellulose membrane. Electrodes without the nylon support become fragile when dry, and they tend to burst at excess pressures of electrolyte when it is pumped against gravity. When electrolyte is flowing through the nylon tube the membrane acts as a permeable barrier permitting the electrolyte to reach the electrophoresis paper without the danger of proteinaceous compounds contaminating the internal electrolyte. The electrophoresis paper is clamped between the electrode and the Lucite support by a split Lucite tube.

This type of electrode arrangement results in uniform potential gradients across the paper, as shown in Fig. 4, for continuous electrophoresis. The provision of pumping the electrolyte against gravity through the electrode extends the uniform part of the electrical field to about 4 cm. below the upper end of the permeable part of the electrode, which remains fairly free of electrolytic gas. Therefore, the useful distance of curtain travel can be extended to about 40 cm. for the 18 by 22 inch paper in addition to the collection tips of 7 cm.

From the Cancer Research Laboratory of the University of Virginia Medical School, Charlottesville, Va.

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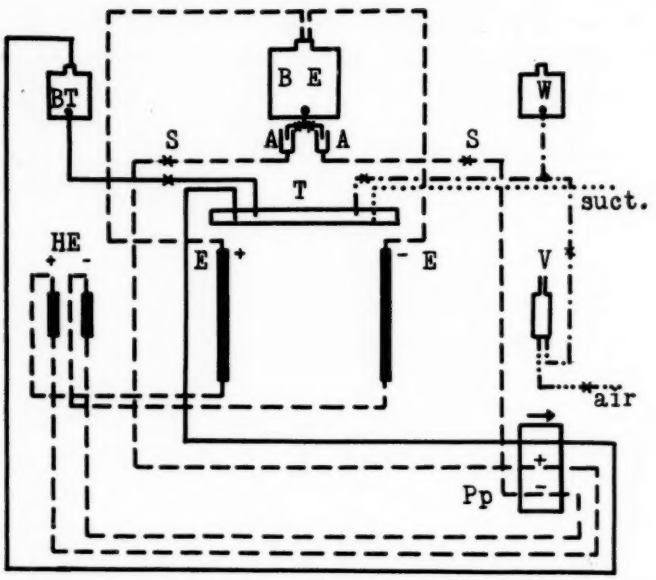


Fig. 1.—Hydraulic circuits, diagrammatic. *A*, adapter of saline infusion set; *BE*, electrolyte reservoir for electrodes; *BT*, buffer reservoir for trough; *E*, electrode; *HE*, heat exchangers; *P*, Pyrex rod; *Pp*, peristaltic pump; *Pr*, electrophoresis paper; *S*, 7-way stopcock, shown expanded; *T*, trough, *V*, vaporizer; *W*, water reservoir; *X*, clamps and stopcocks; ———, trough circuit; — — —, electrode circuit; —.—.—, water supply; ....., drain to aspirator. Fig. 4, *B* translates most of this diagram into actual construction with identical placement of the units.

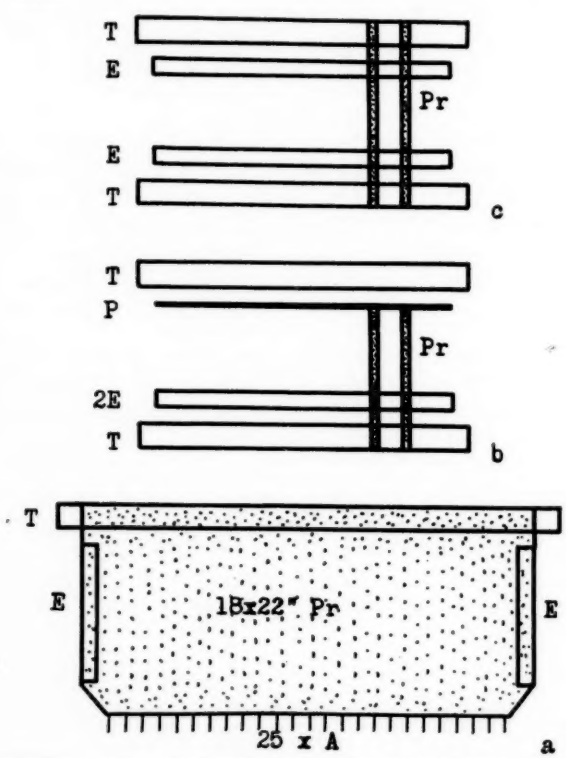
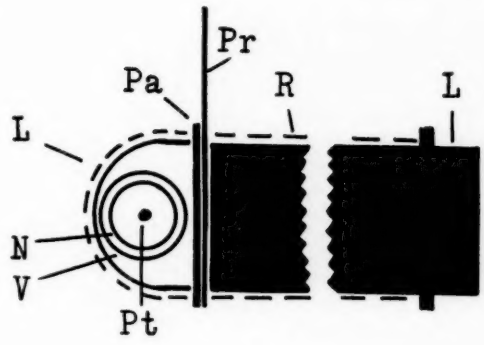
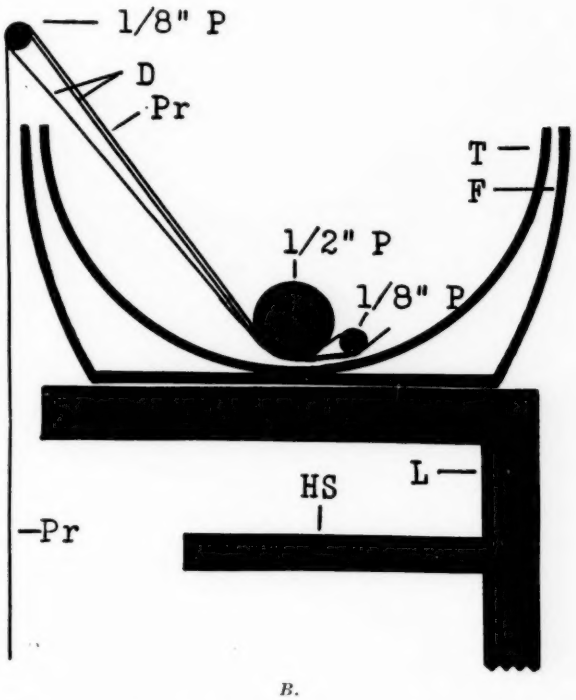


Fig. 2.—Diagrammatic arrangement of electrodes and paper for (*a*) continuous two-dimensional, (*b*) tent-strip separation with the two electrodes behind each other in the horizontal plane, and (*c*) Macheboeuf effect. Symbols as in Fig. 1.



A.



B.

Fig. 3.—*A* and *B*, Diagrammatic construction details of electrodes (*A*), and trough (*B*). (For *C*, see page 138.)

D, Dacron fabric; *F*, trough support; *HS*, support for horizontal electrode or Pyrex rod; *L*, Lucite, 0.5 × 1.75 × 21 inches; *N*, Nylon tube (No. 516-NOO, specially prepared and kindly supplied by Dr. J. S. Tapp, Research Department, Chemstrand Corporation, Decatur, Alabama); *P*, Pyrex rod; *Pa*, heavy absorbent paper, 0.5 inches wide; *Pr*, electrophoresis paper; *Pt*, No. 32 B.&S. gauge Pt wire; *R*, rubber band; *T*, Pyrex trough; *V*, Visking cellulose tubing No. 1215, 0.25 inches.

The electrodes are mounted in a cabinet with a hollow base and back of stainless steel through which coolant is circulating. The electrodes are cooled by the electrolyte buffer, which is chilled by two heat exchangers in the coolant circuit (Fig. 1). Hence, drying and arking from the cellulose envelope is eliminated at potentials up to 1,200 V. A peristaltic pump moves the electrolyte against gravity through the heat exchangers and the electrodes to a 3 liter Mariotte bottle. Here the cathode and anode streams are mixed in order to maintain the pH at the nominal value of the selected buffer. Fresh electrolyte is fed to the pump by splitting the outlet of the Mariotte bottle by a Y-tube leading into two modified drip-adapters, which are on the input side of the pump. The electrical anode and cathode circuits remain isolated as long as the electrolyte is dripping in the adapters.

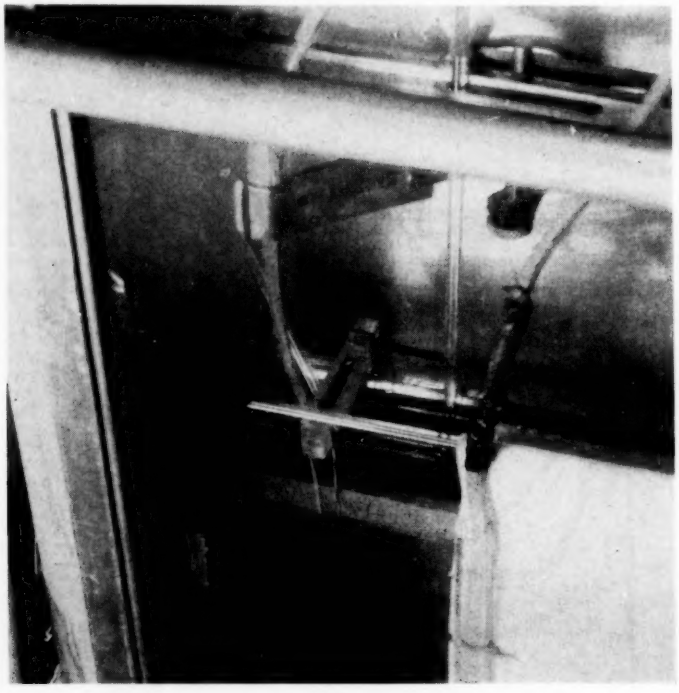


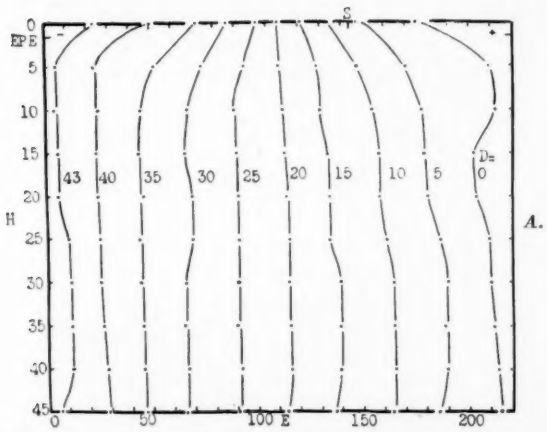
Fig. 3, *C*.—Actual assembly of electrodes and trough shown in Fig. 3, *A* and *B*.

The trough for continuous electrophoresis experiments is supplied with buffer from a 500 ml. Mariotte bottle. The overflow of the trough is taken up by the suction side of the pump and returned to the reservoir. A constant and predetermined level of buffer in the trough is thus maintained. Whenever an experiment is finished, all hydraulic circuits can be flushed with distilled water from another Mariotte bottle feeding through a 7-way stopcock. The same reservoir supplies water to a vaporizer for the maintaining of a saturated atmosphere in the apparatus.

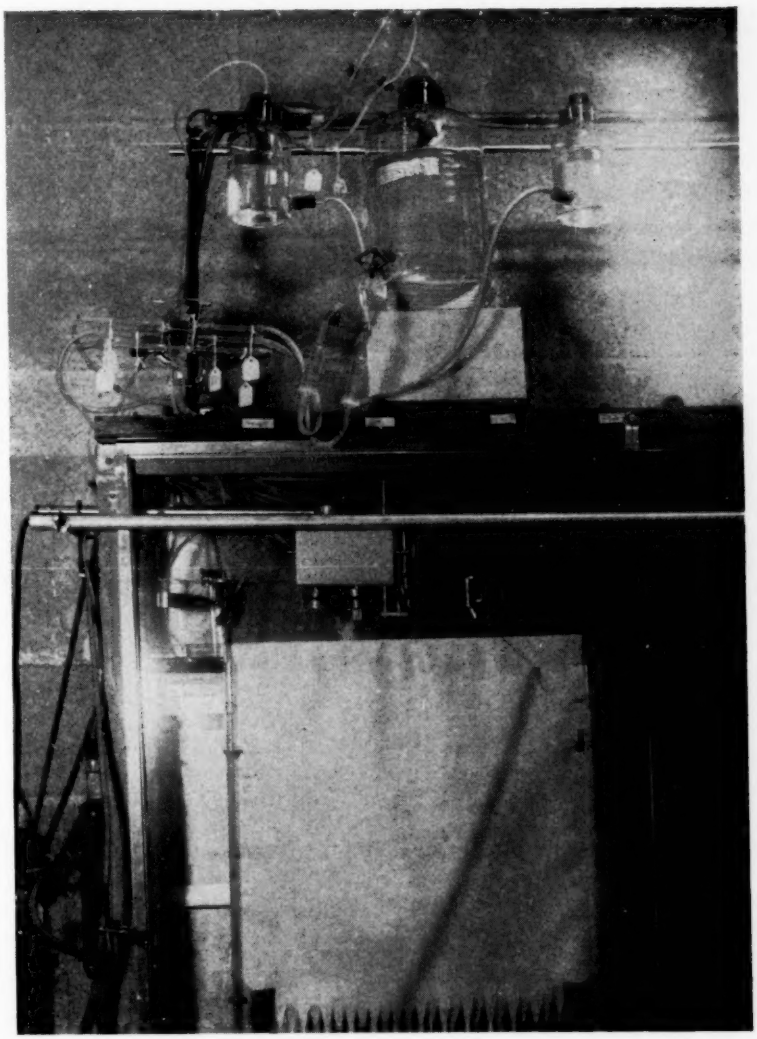
Continuous two-dimensional electrophoresis and strip electrophoresis for the Macheboeuf effect succeed best if the paper is led into the upper trough, as shown in Fig. 3. The paper is led over a 0.125 inch Pyrex rod and a sleeve of a selected, white Dacron fabric downward under a 0.5 inch Pyrex rod in the trough. The fabric serves to equalize and speed up the ascending flow of buffer from the trough to the apex of the paper. It also reduces considerably tearing of the wet paper.

The power supply satisfies the given specifications.* Two power outlets with individual regulation are continuously variable from 125 volts to 1,250 volts D.C. at 30 Ma.

*The power supply was designed and constructed by Mr. Ray C. Williams, Department of Biophysics, Medical College of Virginia, Richmond, Virginia.



A.

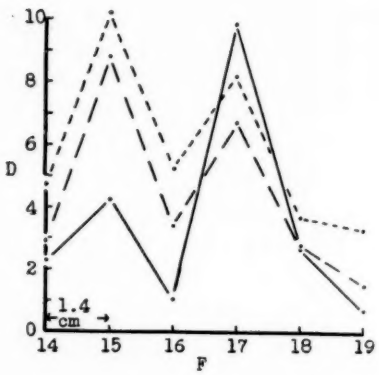


B.

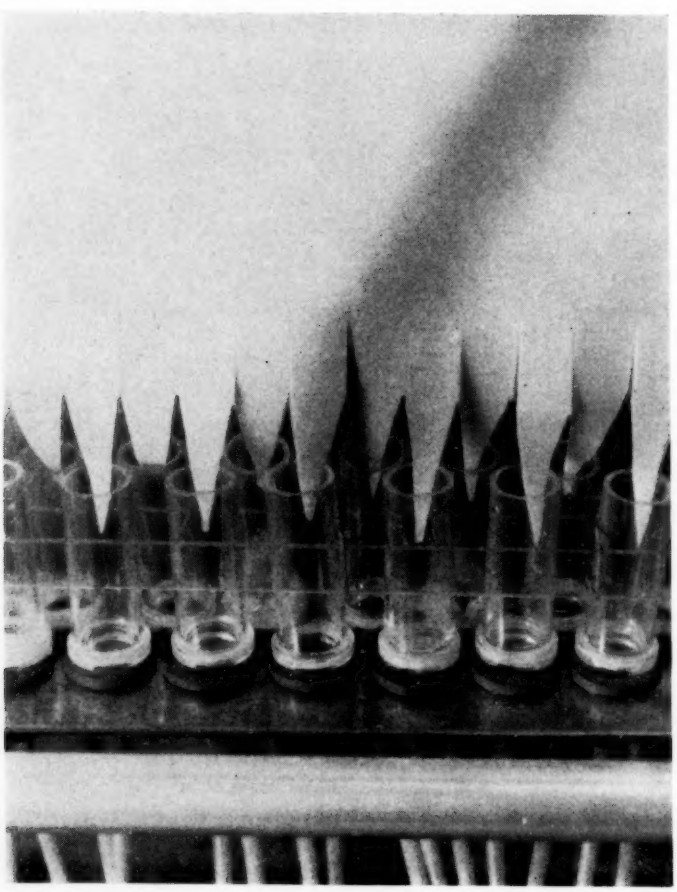
Fig. 4.—Potential distribution on 18 × 22 inch Whatman 3 MM paper as expressed by (*A*) equidistant voltage measurements, and (*B*) a dye. Electrolyte buffer of pH 8.6 and ionic strength 0.025.

For *A:* *E*, potential in D.C. volts; *H*, vertical distance in centimeters from upper suspension of paper; *D*, horizontal distance from center of anode; *S*, location of sample application; *EPE*, end of permeable section of electrodes; potential gradient = 4.97 V/cm.

For *B:* potential gradient = 10.3 V/cm.; load = 13 Ma.; temperature = 13° C; 5.0 ml. of 1.5 × 10^{-4} M bromphenol blue in borate buffer applied at the rate of 0.33 ml./hour.



A.



B.

Fig. 5.—Resolution of continuous 2-dimensional electrophoresis as shown by (*A*) spectrophotometric evaluation of a protein fractionation, and (*B*) visualization of the migration of a dye. To *A:* fractionation of a solution of 0.35 per cent beef serum albumin treated with H_2O_2. Borate buffer pH 8.6; ionic strength 0.025; potential gradient 11.3 V/cm.; load 15.8 Ma.; temperature 17° C.; rate of feeding of 5 ml. of sample 0.33 ml./hour; abscissa in sequence of fractions, ordinate in arbitrary units of optical density; — — —, optical density at 280 mμ; — — — — —, difference of optical densities at 515 and 510 mμ of fractions in fluorescein; — — —, optical density at 350 mμ of suspension of fractions in sulfosalicylic acid. To *B:* migration of bromphenol blue into collectors. See Fig. 4, *B*.

per output. One series of 10 VR-OA2 tubes regulates both output circuits by providing reference and electrode voltages to a 6AU6 error amplifier tube. This arrangement permits a maximum variation of the output potential of <0.45 per cent under an input voltage change from 100 volts to 120 volts. The output is insensitive to load variations. Therefore the power supply can be used for continuously and simultaneously separating a control and an unknown. Samples for continuous fractionations are applied to the paper by a motor-driven syringe,* which is connected to capillary latex tubing and a flexible No. 18 gauge needle. It can be positioned within any location of a 5 cm. wide area along the upper margin of the paper. The applicator pump, which is located in the cooled electrophoresis chamber, can extrude from 0.02 to 0.90 ml. of sample per hour.

An example of continuous fractionation is given in Fig. 5A. It demonstrates two proteinaceous fragments after a reaction between beef serum albumin and hydrogen peroxide, which are resolved by 5 collection tips within a total distance of 5.60 cm. Fig. 5B illustrates the course of a band of bromphenol blue at the collection point where the bulk of the dye is descending into one collector.

SUMMARY

A paper electrophoresis apparatus is described which can be used for continuous two-dimensional separation and for strip experiments. Provisions exist for maintaining constant voltage, temperature, pH of the electrolyte, and sample and curtain flow. A resolution of 1.4 cm. per fraction can be obtained for continuous separations.

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*Supplied by Bender and Hobein, Munich, Germany.

A SIMPLIFIED MODIFICATION OF PIKE'S BROTH

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SYRACUSE, N. Y.

IN A previous communication[1] from this laboratory, it was reported that a broth[2] selective for the isolation of beta hemolytic streptococci could be modified so that the basic medium could be prepared from dehydrated materials. One of the inherent deficiencies of the original medium remains in this modification, namely, the necessity for adding the sodium azide, crystal violet, and sheep blood separately to each tube at the time of inoculation. The subsequent development of a broth[3] that supports the growth of beta hemolytic streptococci exceptionally well in the absence of blood or serum prompted the trial of the additionally modified medium which is being reported at this time. Since no blood is required, the sodium azide and crystal violet can be incorporated into the broth at the time of its original preparation, eliminating another step from the use of Pike's broth.

MATERIALS AND METHODS

Broth.—The formula is as follows:

INGREDIENT	GRAMS
Neopeptone (Difco)	20.0
Myosate (BBL)	10.0
Phytone (BBL)	4.5
NaCl	8.0
Glucose	10.0
Na_2HPO_4	6.0
Na_2CO_3	1.25
Sodium azide	0.075
Crystal violet	0.002

Distilled water is added to a volume of 1 liter and the mixture heated to boiling for five minutes. It is permitted to cool to room temperature, the pH adjusted to 7.8, then filtered through paper, dispensed into tubes or stock flasks, and autoclaved at 12 pounds for 10 minutes. The broth is then ready for use and may be stored in the refrigerator for at least one week.

Blood Agar Plates.—Trypticase soy agar containing defibrinated sheep's blood in 5 per cent concentration.

Cultures.—Sterile cotton swabs used to obtain pharyngeal cultures serve as the inoculum for a direct blood agar plate (BAP) and are then placed in a 16 × 150 mm. tube containing 2.0 ml. of the inhibitory broth. Following overnight incubation, a loopful of the broth is streaked on a BAP and the streptococci subsequently are isolated from this plate.

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Serologic.—The streptococci were serologically* identified according to the method of Swift and associates.[4]

DATA AND RESULTS

The data are derived from 2,619 throat cultures which were seeded directly on a BAP and from the modified broth. Of this group of specimens, 660 (25 per cent) were found to have beta hemolytic streptococci. In Table I is summarized the distribution of the various streptococcal groups which

TABLE I. STREPTOCOCCAL STRAINS OF DIFFERENT SEROLOGIC GROUPS ISOLATED FROM DIRECT BLOOD AGAR AND MODIFIED SELECTIVE BROTH

STREPTOCOCCAL GROUP	BAP ONLY		BROTH ONLY		BOTH		TOTALS	
	NO.	%	NO.	%	NO.	%	NO.	%
A	55	14	73	18	275	68	403	61
B	5	8	12	31	22	56	39	6
C	3	15	8	40	9	45	20	3
D	1	10	4	40	5	50	10	2
G	9	6	86	61	45	32	140	21
N.G.*	10	21	30	63	8	17	48	7
Totals	83	13	213	32	364	55	660	100

*Nongroupable.

were encountered; the majority were members of Group A. Slightly more than half of the total was isolated from both media, but among the remainder, the broth yielded significantly more isolations than did the direct BAP. Certain differences are of interest and these are more readily apparent in Table II in

TABLE II. COMPARATIVE EFFECTIVENESS OF MODIFIED SELECTIVE BROTH AND DIRECT BLOOD AGAR FOR THE ISOLATION OF STREPTOCOCCI OF DIFFERENT SEROLOGIC GROUPS

STREPTOCOCCAL GROUP	BAP		BROTH		TOTALS	
	NO.	%	NO.	%	NO.	%
A	330	49	348	51	678	100
B	27	44	34	54	61	100
C	12	41	17	59	29	100
D	6	40	9	60	15	100
G	54	29	131	71	185	100
N.G.*	18	32	38	68	56	100
Totals	447	44	577	56	1,024	100

*Nongroupable.

which are consolidated the results obtained with the two methods. It will be noted that whereas Group A strains were detected with similar frequencies by both methods, many more Group G's and nongroupable† streptococci were isolated from the broth than from the direct BAP. This also was true of the Group C's and D's, but here the numbers are fewer and the significance of this observation is somewhat doubtful.

The types encountered within Group A are summarized in Tables III and IV. No great differences are noted except that two cultures appear to be better than one, and more of the nontypable strains were isolated from the broth.

*Dr. Elaine L. Updyke of the Communicable Disease Center, U.S.P.H.S. Chamblee, Ga., very kindly has supplied us with the necessary typing sera.

†Some of these could have been of Group F but no serum was available for this group.

TABLE III. GROUP A STREPTOCOCCAL TYPES ISOLATED FROM DIRECT BLOOD AGAR AND MODIFIED SELECTIVE BROTH

GROUP A TYPE	BAP ONLY		BROTH ONLY		BOTH		TOTALS	
	NO.	%	NO.	%	NO.	%	NO.	%
1	8	6	11	8	115	86	134	33
3	20	20	15	15	66	65	101	25
5	6	33	6	33	6	33	18	5
6	0	0	7	19	30	81	37	7
12	0	0	0	0	2	100	2	1
13	3	100	0	0	0	0	3	1
18	0	0	0	0	1	100	1	<1
28	1	20	0	0	4	80	5	1
N.T.*	17	17	34	33	51	50	102	25
Totals	55	14	73	18	275	68	403	100

*Nontypable.

TABLE IV. COMPARATIVE EFFECTIVENESS OF MODIFIED SELECTIVE BROTH AND DIRECT BLOOD AGAR FOR THE ISOLATION OF DIFFERENT GROUP A STREPTOCOCCAL TYPES

GROUP A TYPE	BAP		BROTH		TOTALS	
	NO.	%	NO.	%	NO.	%
1	123	49	126	51	249	100
3	86	52	81	49	167	100
5	12	50	12	50	24	100
6	30	45	37	55	67	100
12	2	50	2	50	4	100
13	3	100	0	0	3	100
18	1	50	1	50	2	100
28	5	56	4	44	9	100
N.T.*	68	44	85	56	153	100
Totals	330	49	348	51	678	100

*Nontypable.

The results obtained with "Pike's" broth made by this new, simplified, nonblood-containing formula are substantially similar to our previously reported experiences.[1] The new medium possesses an additional advantage in that it may be prepared complete in one step and does not require further manipulation prior to use.

The technical assistance of Miss Eleanor Gerhardt is gratefully acknowledged.

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A QUANTITATIVE METHOD OF LYSOZYME DETERMINATION

I. An Investigation of Bronchial Lysozyme

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LYSOZYME, a bacteriolytic and mucolytic enzyme, has been shown to be widely distributed in human secretions and tissues.[1] Its role in various diseases, particularly peptic ulcer and chronic ulcerative colitis, has been the subject of considerable investigation.[2-5] The presence of lysozyme in nasal mucus, sputum, and minced lung tissue[6-9] has led to the speculation that this naturally occurring enzyme might have a protective function in the respiratory tract[10, 11]; however, there have been no reports, to our knowledge, of lysozyme measurements in bronchial secretions. Since this would seem to be of particular importance in the relationship of lysozyme to various respiratory tract diseases, the present study has been undertaken.

The objectives of the study made it desirable to devise a method for measuring and comparing on a dry weight basis the quantity of lysozyme in biologic specimens from different sources.

METHOD

Specimens of sputum, tracheal mucus, and bronchial mucus were collected from groups of patients suffering from pulmonary tuberculosis, chronic bronchiectasis, intrinsic bronchial asthma, carcinoma of the lung, and several diseases not related to the respiratory tract. Among the latter were two patients with chronic ulcerative colitis, and from them stool specimens were obtained. In addition, nasal polyp tissue removed from two asthmatic patients and a pooled sample of the "buffy layer" from six normal blood specimens were studied. All material was collected and treated with use of a modification of previous methods.[6, 8]

Sputum was collected in sputum cups. Tracheal and upper bronchial mucus (5 to 6 cm. beyond the mid-trachea to the primary bronchi) was obtained during anesthesia for various surgical procedures in the following manner. A 50 ml. graduated centrifuge tube was fitted with inlet and outlet glass tubes fastened in a two-hole rubber stopper. A 12 cm. soft rubber catheter was attached to the inlet tube. While constant suction was applied to the outlet tube, the anesthetist collected the specimen (usually about 1 ml. of mucus) by passing the catheter as far down the trachea as possible. After removal of the catheter, exactly 25 ml. of saline (0.9 per cent) was drawn through it to wash out all the contained mucus.

Specimens from the lower bronchial tree (usually to the level of the secondary bronchi) were obtained during bronchoscopy and were treated in the same manner as those obtained through the tracheal tube.

After dilution of the specimen with saline solution, the amount of mucus obtained was determined by subtracting 5 ml. from the total volume. Fifty milliliters of acetone

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was added, and the mixture was agitated for two hours to insure complete precipitation of the lysozyme. In addition, the mixture was refrigerated for 48 hours at 5° C. It was then centrifuged at high speed (3,500 r.p.m. for 20 minutes) in a clinical centrifuge and the supernatant was decanted. This was repeated three times. The residue remaining after the third centrifugation was transferred to a weighing dish along with a small portion of acetone rinse. This was air dried and brought to a constant weight in a vacuum oven (55° C.). The dry weight was recorded and the material quantitatively transferred into a centrifuge tube, with use of about 35 ml. of buffer solution as a rinse. The buffer solution used throughout the entire procedure was a standard phosphate buffer which insured a constant pH of 6.2 (Difco-Bacto-lysozyme buffer). This was prepared in 1 L. amounts with distilled water and stored in a refrigerator. In order to get all of the lysozyme into the buffer solution, it was necessary to agitate the residue in small amounts of buffer (10 to 20 ml.) for several hours and to repeat the agitation in fresh buffer solution until the residue had been washed three times. The final volume of the buffer extract of the sample was brought to 100 ml. with additional buffer solution.

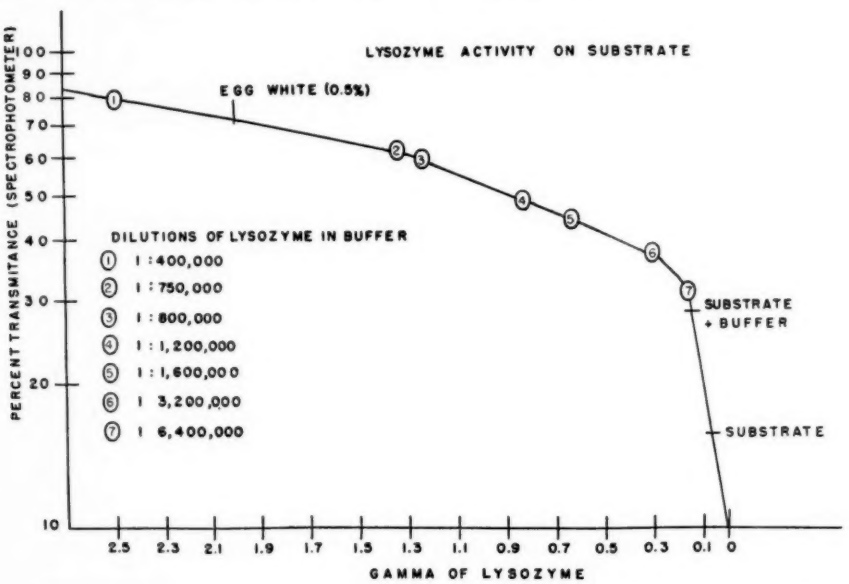

Fig. 1.—Standard curve prepared by 41 separate determinations of standard lysozyme action on the substrate. The lysozyme concentration of the unknown was read from this curve.

Analysis of the amount of lysozyme activity was accomplished in the following manner:

1. All solutions were brought to room temperature and all light transmission changes were recorded on a Coleman Universal spectrophotometer (Model 14, setting at 540 μ, with distilled water for the 100 per cent transmission). A standard substrate solution was prepared in 1 L. quantities with use of distilled water and an ultraviolet-killed, dried culture of *Micrococcus lysodeikticus* (Difco-Bacto-lysozyme substrate). Aliquots of the buffer extract of the lysozyme sample were diluted 1:20, 1:50, 1:100, 1:200, 1:1,000, and 1:2,000. Exactly 5 ml. of each of these sample dilutions was added to 5 ml. of the substrate solution in special spectrophotometer tubes. These mixtures were incubated at room temperature for exactly 20 minutes. The action of the lysozyme on the substrate produced a lessening of the turbidity of the original substrate solution. This turbidimetric change was recorded as percentage of transmission. The light transmission of the

substrate solution was recorded during each analysis and was found to be quite constant. A solution of 5 ml. substrate solution plus 5 ml. of buffer was found to also have a constant light transmission.

2. Standard dilutions of a known lysozyme preparation (Difco lysozyme) crystallized from fresh egg white was prepared as previously described.[13] Dilutions were made up in the buffer solution and in the proportions indicated in Fig. 1. Exactly 5 ml. portions of the known lysozyme dilutions were then incubated with the substrate and read exactly as the unknown lysozyme samples. This was done at the time of each sample analysis. An average of 41 known lysozyme readings was used to plot the curve shown in Fig. 1. This was considered as a standard curve with percentage of transmission plotted against the amount of lysozyme in gamma, as determined by the known lysozyme dilutions. The widest difference between the average of all determinations of the known lysozyme dilutions and the median of these values was 4.2 per cent transmission with an average difference of 2.2 per cent. Complete lysis or clearing of the substrate with an excess of standard egg white gave a reading of 90 per cent transmission on repeated determinations.

3. The percentage of transmission of the unknown samples was plotted on the standard curve and recorded as gamma of lysozyme per milliliter of buffer extract and gamma of lysozyme per milligram of dried mucus (see tables).

This method of assay is a modification of the method of Goldsworthy and Florey[8] recommended by Armour and Company. Results were expressed in metric units, in accordance with previous suggestions.[14, 15]

RESULTS AND DISCUSSION

The results of quantitative lysozyme determinations in specimens obtained from the respiratory tract of 3 patients are shown in Tables I and II. In the majority of instances, several determinations were made on each specimen on different days. These repeated measurements were entirely consistent. All specimens showed some amount of lysozyme activity, but there was a wide range which did not seem to relate to any specific disease process in the relatively small group studied. Results in control patients did not differ from those in patients with respiratory tract diseases as far as comparisons were possible.

1. Five patients with intrinsic asthma (Table II, Patients 2 to 5) were also studied for saliva lysozyme activity. One to two milliliters of saliva had a dry weight ranging from 6 to 297 mg. The average lysozyme concentration was 9.8 γ per milliliter extract (0.03 to 23.3) or 10.6 γ per milligram dry saliva (0.4 to 22.0). These values were slightly higher than the tracheal specimens and several were higher than the maximum lysozyme tracheal value. Nasal polyps from 2 intrinsic asthma patients were fresh ground and extracted with cold saline. The lysozyme activity of both specimens was 0.1 γ lysozyme per milligram dry material and was the same order of activity as the bronchoscopic specimens. The majority of specimens obtained from the trachea and upper bronchi showed significant amounts of lysozyme. In the collection of the specimens, every effort was made to eliminate the possibility of contamination with saliva. With but one exception, specimens of mucus obtained from the lower respiratory tract (secondary bronchi) of patients with chronic pulmonary tuberculosis and intrinsic bronchial asthma were found to contain minimal quantities of lysozyme.

In the specimens of colon mucus from patients with chronic ulcerative colitis there was considerable difference in the lysozyme content between the acute stage of the disease and during a remission. One milliliter of mucus stool from

TABLE I. LYSOZYME CONTENT OF TRACHEAL TUBE SPECIMENS*

PATIENT	CLINICAL DIAGNOSIS	MUCUS (ML.)	DRY WEIGHT OF SPECIMENS (MG.)	γ LYSOZYME PER ML. OF ETXRACT	γ LYSOZYME PER MG. OF DRY MUCUS
6. J. B.	Duodenal ulcer	1.5	17.6	0.16	0.1
7. J. N.	Breast tumor	1.5	17.6	0.33	0.2
8. B. W.	Ruptured lumbar disc	1.0	53.0	0.31	0.3
9. V. K.	Carcinoma of parotid	1.0	6.6	0.07	1.0
10. M. S.	Duodenal ulcer	2.0	79.9	8.7	10.9
11. M. V.	Thyroid adenoma	1.0	70.5	8.8	12.5
12. L. D.	Carcinoma of colon	1.5	95.7	6.9	7.4
13. M. C.	Pulmonary valvulotomy	0.5	12.1	0.86	6.9
14. V. K.	Carcinoma of rectum	3.0	205.2	11.5	5.6
15. C. D.	Carcinoma of lung	1.0	24.3	2.4	4.9
16. L. H.	Carcinoma of lung	1.0	38.6	0.66	1.7
17. L. H.	Pulmonary tuberculosis	1.0	15.1	1.27	15.1
18. V. W.	Pulmonary tuberculosis	1.0	5.2	0.34	6.5
19. L. L.	Pulmonary tuberculosis	2.0	107.2	5.1	4.7
20. D. K.	Pulmonary tuberculosis	1.0	25.9	2.3	9.0
21. K. F.	Pulmonary tuberculosis	0.5	46.3	1.94	4.2
22. E. D.	Pulmonary tuberculosis	1.0	19.9	0.11	0.1
23. M. K.	Bronchiectasis	1.0	39.7	0.02	0.1
24. L. M.	Bronchiectasis	1.0	10.2	0.03	0.3
25. D. M.	Bronchiectasis	1.0	69.1	1.8	2.6
26. C. K.	Intrinsic asthma	2.0	7.4	0.82	11.0

*Specimen sucked through catheter inserted 5 to 10 cm. beyond endotracheal tube during surgical anesthesia.

a patient with acute ulcerative colitis was 343 mg. dry weight and showed 3.3 γ lysozyme per milliliter extract or 1.0 γ lysozyme per milligram dry material. The stool from a patient in remission showed 0.1 γ lysozyme per milligram dry material. Interestingly enough, when measured on a dry weight basis, the amounts of lysozyme in either case were considerably smaller than in the sputum and upper bronchial secretions of patients with various types of diseases.

Procaine or pontocaine did not seem to influence existing lysozyme activity. Active saliva specimens incubated with these anesthetic agents gave the same results as when tested in their original stage. On the other hand, specimens collected in alcohol were rendered completely inactive. Specimens could be

kept in acetone at 4° C. for several months without deterioration of lysozyme activity. Stock solutions of lysozyme in buffer began to deteriorate after three to four weeks of storage at 4° C.

TABLE II. LYSOZYME CONTENT OF BRONCHOSCOPIC SPECIMENS

PATIENT	CLINICAL DIAGNOSIS	MUCUS (ML.)	DRY WEIGHT OF SPECIMENS (MG.)	γ LYSOZYME PER ML. OF EXTRACT	γ LYSOZYME PER MG. DRY MUCUS
27. I. H.	Pulmonary tuberculosis Diabetes mellitus	1.0	13.4	0.03	0.2
28. I. S.	Pulmonary tuberculosis	1.0	10.3	0.03	0.3
29. E. N.	Diabetes mellitus	1.0	29.9	0.03	0.1
30. M. H.	Pulmonary tuberculosis	1.0	11.7	0.04	0.3
31. M. H.	Intrinsic asthma	1.0	29.4	0.03	0.1
2. M. B.	Intrinsic asthma (rt.)	0.5	12.0	0.07	0.6
2. M. B.	Intrinsic asthma (left)	0.5	23.9	0.07	0.3
3. W. A.	Intrinsic asthma	1.0	13.2	0.03	0.2
4. E. W.	Intrinsic asthma	1.0	20.9	0.03	0.1
5. R. J.	Intrinsic asthma	1.5	97.3	3.8	4.0

As shown by the dry weights of the various specimens in the tables, 1 ml. of mucus was an adequate amount for testing. Specimens of this quantity were readily obtained through an endotracheal tube or bronchoscope. A pool of the buffy layer from 6 normal blood samples was 0.5 ml. and 184 mg. (dry weight). There was 1.2 γ lysozyme per milliliter extract or 0.1 γ lysozyme per milligram dry material. Two milliliters of chicken egg white produced 246 mg. dry material. The activity of this on repeated determinations was 4.4 γ lysozyme per milliliter extract or 1.8 γ lysozyme per milligram dry material.

The expression of lysozyme activity or content on a dry weight basis seemed to have definite advantages over other methods currently in use, since it provided a means of accurate standardization and a resulting better quantitative comparison of the amounts of the enzyme in various body secretions and excretions.

CONCLUSIONS

1. A method of quantitatively determining lysozyme on a dry weight basis is reported.

2. With use of this method, the quantity of lysozyme in specimens of saliva, tracheal and bronchial mucus, nasal polyps, and colon mucus from 33 patients suffering from various diseases has been compared.

3. Saliva and most tracheal specimens contain appreciable lysozyme activity as compared to standard lysozyme and chicken egg white. Bronchial specimens were low in lysozyme content.

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THE USE OF CHICKEN ANTISERUM FOR THE RAPID DETERMINATION OF PLASMA PROTEIN COMPONENTS

I. The Assay of Human Serum Albumin and Gamma Globulin

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INTRODUCTION

THE immunochemical method of antigen assay can provide an accurate and rapid means for quantitating plasma protein components. Routine clinical use of the method, however, has been largely prevented by the difficulties encountered in producing high-titered specific antisera. Rabbits, horses, and, on occasion, chickens have been employed for antibody production. In this regard Goodman and associates[1] observed that in isotonic saline (0.9 per cent NaCl) chicken antiserum in contrast to mammalian yielded very incomplete reactions. A high NaCl concentration of at least 8 per cent was needed to effect complete precipitation. Recent investigations,[2-6] employing titrations based on this observation, have revealed the powerful precipitin response of chickens to antigenic stimuli. Even to poor antigens such as human hemoglobin[2] and orosomucoid,[6] reliable antisera could be produced with relative ease. In view of these results it seemed feasible to work out an inclusive system for the quantitation of human plasma proteins by the use of chicken antiserum. The current studies with antialbumin and antigamma globulin sera represent our first effort in this direction.

The antibody excess zone reaction of an antiserum with antigen provides the calibration curve for estimating the antigen contents of suitably diluted samples. In this zone the amount of precipitation bears a quantitative relationship to the amount of added antigen and for the rabbit system can be described by suitable equations.[7] The empirical calibration curves employed in our studies, as in similar ones by Chow,[8] related the turbidity of the antibody-antigen precipitates to antigen concentration. A close correspondence between the turbidity and protein content of such precipitates has previously been demonstrated.[1, 9]

For the measurement of turbidity two rapid independent procedures were worked out, one adapted to spectrophotometers and the other to nephelometers.

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The nephelometric method, based on light scattering by the antigen-antibody particles, had the advantage of great sensitivity and required less antiserum per analysis. A large number of human sera were assayed for albumin and gamma globulin both immunochemically and by the paper electrophoresis method now finding wide use in clinical laboratories. A close correspondence of results was obtained, and in terms of simplicity of technique and economy of equipment used the immunochemical method offered clear advantages.

MATERIALS AND PROCEDURES

The proteins used for immunization were purified Fr V[10]* and Fr II.[11]* Each by electrophoresis† had less than 2 per cent contaminants. In addition crystalline human serum albumin‡ (HSA), Fr I,* Fr III,* Fr IV-I,* Fr IV-4,* and Fr VI* were available for studies concerned with elucidating the specificity of the antisera.

The antisera were produced in 6- to 12-month-old hens of various breeds. Each bird received a single intramuscular injection of the antigen (at 2 to 5 per cent concentration) thoroughly mixed with an equal volume of aluminum hydroxide gel. In most cases the dosage was 100 mg., but preliminary experiments have indicated that a more powerful response can be obtained with 200 mg. However, the larger dosage enhances the chance of producing antibodies to contaminants in the immunizing preparations. Ten days after the injections the birds were bled by cardiac puncture.§ Following an 18- to 24-hour fast, citrated blood was collected and processed according to the procedure of Silberberg and associates.[6]

The serologic reactions were routinely carried out in medium of 8 per cent NaCl, which in addition contained 0.01 M sodium phosphate buffer at pH 7.6 and 0.01 per cent merthiolate. The antigen solutions and agar antiserum mixtures used in the Oudin tests[12] were also made up to possess this same salt composition. In our routine use of the Oudin procedure for the qualitative detection of the number of migrating antigen-antibody bands, the reaction tubes were incubated at 38° C. for three days before inspection.

The protein contents of the antigen standards and of the human serum samples were determined by the biuret reaction with the reagent of Gornall.[13] The samples under analysis were diluted to contain between 0.5 and 1.5 mg. of protein per milliliter. To 0.5 ml. volumes of these diluted samples equal volumes of the biuret reagent were mixed. The buffered 8 per cent NaCl solution was used both as the diluent and reagent blank. The mixtures in 10 × 75 mm. calibrated tubes were incubated at 38 degrees for 15 minutes, and their optical densities were determined in the Junior Coleman spectrophotometer at 540 mμ. A solution of human serum albumin of known nitrogen content served as the standard.

Paper electrophoretic determinations were performed by the method of Durrum[14] as adapted for use with the Spinco Model R electrophoresis system. A glass plate pressure method of paper electrophoresis was also employed to detect differences in the mobilities of the gamma proteins in certain multiple myeloma serum samples.‖

RESULTS

Determination of Antiserum Specificity.—By a variety of testing procedures it was established that each batch of antialbumin and antigamma globulin sera produced by the 100 mg. dosage was for practical purposes specific

*These fractions were generously provided to us by Dr. H. L. Taylor of the Blood Fractionation Laboratory of the Michigan Department of Health.

†We are indebted to Mr. Abner Robinson of Children's Hospital for performing the moving boundary electrophoretic analyses.

‡Originally produced by Squibb and Company and generously given to us by Dr. R. K. Jennings.

§Brown and Wolfe[3] and Abramoff and Wolfe[5] have demonstrated that very potent antisera to albumin and gamma globulin can be produced in 5- to 6-month-old roosters by single intravenous injections of antigen without adjuvant at a dosage of 40 mg. per kilogram of body weight. By this procedure the optimal time for bleeding was 8 days after injection.

‖We are indebted to Dr. R. M. Johnson for carrying out these determinations.

for its homologous antigen. Strong precipitin reactions occurred with the homologous antigen and failed to take place to a significant degree with other components of human serum. By the Oudin procedure only a single band could be demonstrated for the reaction of human serum with each of the antisera. The various Cohn fractions were also reacted against the antisera. For example, with antialbumin serum Oudin tests were run with Fr VI, V, IV-4 and IV-I. Fractions VI, IV-4, and IV-I have as major components the most likely contaminants of an albumin immunizing preparation. In confirmation of the antiserum's specificity only one band, due to the albumin in these preparations, developed in each test.

The specificities of the antisera were further investigated by absorption techniques. In Table I an experiment is presented which demonstrates that when just enough crystalline HSA was used to remove all or all but a trace of the antibodies to albumin in an anti-HSA serum, the reactions previously observed with human serum and with the Cohn fractions known to contain albumin disappeared due to the removal of the antibodies to albumin. Similarly with the appropriate antiserum when just enough Fr. II was used to remove all but a trace of antibodies to Fr. II, the reactions with human serum and with the Cohn fractions known to contain gamma globulin disappeared.

TABLE I. DEMONSTRATION OF SPECIFICITY OF ANTI-HSA SERUM (P-6) FOR CRYSTALLINE HSA BY SUPERNATANT TESTING

MCG. CRYST HSA IN EACH ORIGINAL REACTION*	EXCESS ANTI-GEN†	EXCESS ANTIBODY‡									
		MCG.		MCG.		MCG.		MCG.		MCG. FR VI	NORMAL HUMAN SERUM
		CRYST	HSA	FR	IV-4	FR	IV-1	FR	III		
		1	10	1	10	1	10	1	10	4	0.001 ML.
180	−	+	+	±	+						
218	±	−	+			−	±	−	−		
270	+(8.6)	−	+	−	±					−	+
360	+(20.7)	−	−	−	−	−	−	−	−	−	−

*One milliliter P-6 + 1 ml. antigen solution. After 3-hour incucation at 38° C. precipitate removed by centrifugation.

†Six-tenths milliliter supernatant + 0.5 ml. ⅔ P-6. The number in parentheses represents estimated mcg. HSA in the supernatant.

‡Two-tenths milliliter supernatant + 0.1 ml. antigen solution.

In another type of experiment a partial absorption of a portion of a representative antiserum was carried out with a protein fraction containing a large amount of the contaminants of the immunizing preparation and a smaller amount of the main immunizing antigen. The amount of this antigen in the absorbing fraction was then assayed by both the absorbed and unabsorbed antiserum. Identical assays were obtained by antisera shown to be specific for a single antigen by the Oudin procedure. The practical reliability of these antisera was thereby demonstrated. A typical experiment is recorded in Table II.

Rapid Turbidimetric Procedures for Immunochemical Assay.—Two rapid turbidimetric procedures of assay were worked out, one adapted to spectrophotometers and the other to nephelometers.

TABLE II. DEMONSTRATION OF SPECIFICITY OF ANTI-HSA SERUM (P-6) FOR CRYSTALLINE HSA BY PARTIAL ABSORPTION WITH FR IV-4

MCG. FR IV-4 OF EACH SAMPLE	MCG. HSA FOUND IN EACH SAMPLE BY SPECTROPHOTOMETRIC METHOD		% HSA IN EACH SAMPLE		AVERAGE % HSA IN FR IV-4	
	A	B	A	B	A	B
6.8	0	0				
13.5	3.5	3.3				
27.0	11.0	10.5	40.8	38.9		
54.0	24.5	23.0	45.4	42.6	43	41
108.0	46.0	45.5	42.6	42.2		
216.0	92.5	87.0	42.8	40.3		

A, For every 1 ml. P-6, 133 mcg. Fr IV-4 used for absorption.

B, Serves as the control for *A*. On the basis of an original determination of 43 per cent HSA in Fr IV-4 by unabsorbed P-6, 57 mcg. cryst. HSA for every 1 ml. P-6 used for the control absorption.

In the spectrophotometric procedure a Junior Coleman spectrophotometer and calibrated cuvettes requiring a 1.0 ml. minimum volume were employed. The first step in carrying out the albumin assay was to make a 1/100 dilution in 8 per cent saline of each human serum sample. A 0.1 ml. of each diluted sample was pipetted into a separate cuvette. Albumin standards, ranging from 6 to 60 mcg. per 0.1 ml., were also pipetted into cuvettes. The reagent blank cuvette contained a 0.1 ml. of the 8 per cent saline. Then 1 ml. of a fixed dilution of the antihuman albumin serum was added to each cuvette.* The antibody-antigen reactions developed rapidly, and their opacities were recorded after 15 minutes' incubation at room temperature. Little change in the character of the turbidity occurred between 15 and 20 minutes, but after 20 minutes, floccules began to settle out. The opacities of each standard and sample were read at 450 mμ against the reagent blank cuvette (antiserum + 8 per cent NaCl). A calibration curve relating optical density (O. D.) units of turbidity to micrograms of albumin was plotted, and the turbidities of the samples were converted into known amounts of albumin.

The reactions afforded by normal samples at 0.001 ml. fell on the upper portion of the calibration curve. Since albumin levels are always maximal in normals, the full-span of the calibration curve was available for measuring the abnormal levels encountered in pathologic specimens. For the occasional sample of heavily hemolyzed serum which still possessed visible color at the 1/100 dilution, the true O.D. afforded by the turbidity of the antigen-antibody reaction could be obtained by subtracting from the total O.D. of the reaction mixture the O.D. afforded by a 0.001 ml. of the sample diluted up to 1.1 ml. with 8 per cent saline. In the latter case the reagent blank was, of course, 8 per cent saline. The validity of the above procedure was demonstrated by the experiment recorded in Table III in which the reactions of an antialbumin serum were determined in the presence and absence of rat hemoglobin.

*Calibrated automatic syringe pipettes were used to facilitate the delivery and rapid mixing of solutions. For volumes of 2 ml. or less the Cornwall pipette was employed, and for delivery of larger volumes, such as encountered in the nephelometric method, the 5 ml. size syringe pipette of Northern Tool and Instrument Company (Flushing, N. Y.) proved satisfactory.

The antisera used for assay purposes were adjusted by dilution with 8 per cent saline to comparable potencies so that their maximal precipitation with antigen yielded turbidities of about 50 per cent transmittance in volumes of 1.1 ml. Maximal precipitation occurred in the antigen excess zone, and 200 to 400 mcg. of antigen was employed per test in effecting the potency adjustments. The amount of undilute antiserum required to give the 50 per cent transmittance ranged from 0.1 to 0.4 ml. for the various antialbumin and anti-gamma globulin sera.

TABLE III. DEMONSTRATION THAT THE EFFECT OF HEMOGLOBIN (HGB.) CAN BE CORRECTED FOR IN THE SPECTROPHOTOMETRIC ASSAY OF ALBUMIN

CONTENTS OF EACH 1.1 ML. REACTION TUBE	O.D.† 450 Mμ	CORRECTED O.D.‡ 450 Mμ
1. 0.333 ml. P-6, 8% NaCl	0.000	
2. 0.333 ml. P-6 + 24 mcg. HSA, 8% NaCl	0.110	0.110
3. 0.333 ml. P-6 + 48 mcg. HSA, 8% NaCl	0.183	0.183
4. 0.333 ml. P-6 + 24 mcg. HSA + 50 mcg* Rat Hgb. 8% NaCl	0.143	0.110
5. 0.333 ml. P-6 + 48 mcg. HSA + 50 mcg. Rat Hgb., 8% NaCl	0.217	0.184
6. 8% NaCl	0.000	
7. 24 mcg. HSA + 50 mcg. Rat Hgb., 8% NaCl	0.033	
8. 48 mcg. HSA + 50 mcg. Rat Hgb., 8% NaCl	0.033	

*In a test reaction 50 mcg. Hgb. is equivalent to 50 mg. Hgb. for an undilute sample, since the amount of human serum used in the albumin determination is only 0.001 ml.

†Tube 1 served as the blank for Tubes 2, 3, 4, and 5; Tube 6 served as the blank for Tubes 7 and 8.

‡O.D. of Tubes 7 and 8 subtracted from Tubes 4 and 5 to obtain final O.D.

In our other turbidimetric method a Coleman Model 7 photo-nephelometer and calibrated cuvettes requiring a 5.5 ml. minimum volume were employed. This method, based on light scattering by the antibody-antigen particles, had the advantage of far greater sensitivity. With one-half the quantity of antiserum and five times the reaction volume of the spectrophotometric method, antibody excess zone reactions covering the full breadth of the nephelos scale were obtained. Only one-third the quantity of sample (0.5 ml. of 1/1500 dilution) was used in the albumin assay. To this was added 4.5 ml. of 8 per cent NaCl, and then a 0.5 ml. of the fixed dilution of antiserum. Each reaction was incubated for 20 minutes at 38° C., at which time it reached completion in terms of light scattered by the formation of the antibody-antigen particles. The galvanometer units of turbidity were then recorded. A convenient way for doing this was to first adjust the instrument by the null procedure with the permanent Coleman nephelos standard of highest turbidity. Next with the reagent blank cuvette (antiserum + 8 per cent saline) the galvanometer reading was set at zero with the "Bal" knob. Sample and standard turbidities could then be directly read from the galvanometer scale. Flocculation and settling out of particles was slow to develop due to the high dilution of the system, and readings could be duplicated for at least an additional 45 minutes after removal of the reaction tubes from the water bath. It was necessary, however, to periodically reset the nephelometer with the nephelos standard and the reagent blank cuvette.

Calibration curves obtained on successive assays were remarkably similar. In this regard a composite curve for reactions over a four-month period is shown in Fig. 1. On repeat analyses of human serum samples by the nephelometric procedure the coefficient of variation* was only 3 to 3.5 per cent, whereas by the spectrophotometric procedure it was 5 to 6 per cent. Within the limits of error of the methods identical assays for a sample were obtained by either method and by different batches of antialbumin serum.

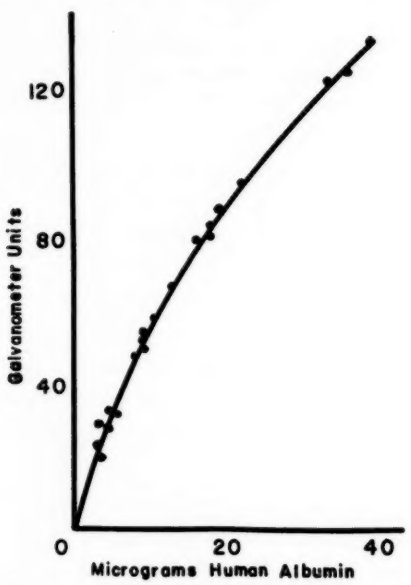

Fig. 1.—Coincidence of repeated antibody excess zone reactions with use of the nephelometric method over a four-month period. Chicken antihuman albumin (P-6) versus human albumin.

The procedure worked out for the assay of human gamma globulin by the spectrophotometric method was similar to that used in the assay of albumin. Reactions were set up with 0.4 ml. and 0.2 ml. quantities of the 1/100 sample dilutions. Seven-tenths milliliter portions of the fixed dilution of antihuman gamma globulin serum were mixed with these aliquots, and total reaction volumes were maintained at 1.1 ml. Opacities were again read at 450 mμ in the Junior Coleman after 15 minutes' incubation at room temperature. A typical calibration curve obtained with Fr II is presented in Fig. 2. It can be seen that the reaction of a normal sample containing at 0.002 ml. 26 meg. of gamma globulin fell at the top of the lower third of the calibration curve. Two thousandths milliliter would seem a good choice for a fixed amount of human serum to be used in the routine assay of gamma globulin, in that the amount of gamma globulin in a pathologic specimen may be either depressed or markedly

*Coefficient of variation = $\frac{100 \times \text{Std. dev. diff.}}{M}$

M = mean protein concentration.

Std. dev. diff. = $\sqrt{\frac{\Sigma (x_1 - x_2)^2}{N}}$

elevated from the amount in normal serum. At 0.002 ml. the undilute sample could have as much as 60 mg. per milliliter of gamma globulin and still yield a reaction encompassed by the calibration curve. In our studies quantities of sample smaller than 0.002 ml. were used in those few instances in which the original reactions fell at the top of the calibration curve and for certain rare multiple myeloma sera in which the differences between a low albumin and high total protein suggested the presence of more than 60 mg. of gamma globulin per milliliter of undilute sample.

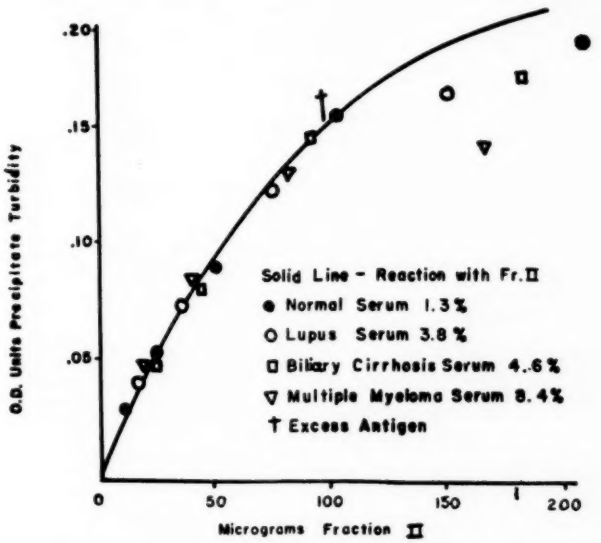

Fig. 2.—Relationship of reactions afforded by serial dilutions of various samples of human serum to the reaction afforded by Fr II with anti-Fr II serum (P-7). The recorded gamma globulin values in grams per cent for the samples were calculated from the antibody excess zone reactions that developed at the appropriate sample dilutions.

The nephelometric method was also used for the assay of gamma globulin. The quantity of antiserum and sample required were less than for the spectrophotometric method, but the sensitivity and accuracy of determination greater as in the albumin assay. Incubation times and manipulative procedures were the same as those worked out for the albumin assay.

Comparison of Immunochemical Assays With Electrophoretic Determinations.—The albumin and gamma globulin contents of the sera of 45 normal adult laboratory workers were determined by paper electrophoresis and by the immunonephelometric procedure. The immunochemical values were recorded both in units of absolute concentration (gram per cent) and relative concentration (per cent of total protein). For calculation of the latter the total protein of each sample was first determined by the biuret method previously described. Table IV compares the mean immunochemical and paper electrophoresis values of our series with moving boundary electrophoresis values for normals obtained by other investigators. The immunochemical value for albumin falls in the middle of the moving boundary values and for gamma

TABLE IV. ALBUMIN AND GAMMA GLOBULIN LEVELS IN NORMAL HUMAN SERUM

INVESTIGATOR	TYPE OF INVESTIGATION	NUMBER OF SUBJECTS	TOTAL PROTEIN (GM. %)	ALBUMIN		GAMMA GLOBULIN	
				ABS. CONC. (GM. %)	REL. CONC. (%)	ABS. CONC. (GM. %)	REL. CONC. (%)
Present experiments	Immuno.	45	7.63 ± 0.44	4.59 ± 0.35	60.1 ± 3.3	1.20 ± 0.18	15.7 ± 2.0
Present experiments	P. elect.	45			53.6 ± 4.4		17.5 ± 3.1
Dole[15]*	M. B. elect.	15			62.3		13.5
Cooper and coworkers[16]	M. B. elect.	13			60.0 ± 2.9		14.9 ± 2.7
Armstrong and coworkers[17]*	M. B. elect.	20	7.50†	4.30	57.3	1.10	14.7
Taylor and coworkers[18]	M. B. elect.	30			63.0 ± 2.8		12.9 ± 2.0
Reiner and coworkers[19]	M. B. elect.	60	7.22 ± 0.48	4.10 ± 0.32	56.8 ± 3.0	1.00 ± 0.20	14.4 ± 2.4
Von Frijtag Drabbe and Reinhold[20]	M. B. elect.	65	7.36	4.10	55.7	1.13	15.4
Sehon and coworkers[21]	M. B. elect.	22 ♂	7.93 ± 0.56	4.73 ± 0.31	59.5 ± 3.2	1.08 ± 0.19	14.3 ± 2.5
		14 ♀	7.92 ± 0.71	4.69 ± 0.39	58.4 ± 2.3	1.08 ± 0.17	14.2 ± 1.8

Immuno. = Immunochemical assay.
P. elect. = Paper electrophoresis assay.
M. B. elect. = Moving boundary electrophoresis assay.
*Plasma values converted into serum values by subtracting three-fifths of the phi area from the total and considering the other two-fifths fast-moving gamma globulin.[22]
†Absolute values taken from Hughes.[23]

globulin is slightly larger. Within our series the correspondence of immunochemical and paper electrophoresis values was also good, but not as close. The immunochemical values for albumin tended to be about 11 per cent greater than the paper electrophoresis values and for gamma globulin 11 per cent smaller. These discrepancies may in part be explained by the trailing of albumin that occurs in paper electrophoresis. The amount of trailing has been shown[24] to be fixed rather than proportionate. This helps explain the data presented in Table V for representative normal and pathologic sera. It can be seen that in the samples possessing depressed albumin levels, the immunochemical values are on the average 15 per cent higher than the electrophoretic values. However, the essential correspondence of the two methods in the assay of albumin and gamma globulin is clearly evident.

The only failure of correspondence encountered in our studies was in the gamma globulin assay of Hun, a specimen from a patient with macroglobulinemia. Electrophoretically a large quantity of a fast moving gamma component was found in the sample, but just a trace reaction developed with the antigamma globulin serum. Even at that, the presence of a large quantity of protein bearing some relationship to normal gamma globulin could be suspected from the immunologic results in that the trace reaction was obtained at very high dilution of the sample. The myeloma sera of the gamma type yielded extensive cross reactions with the antiserum to Fr II, and as a consequence their myeloma globulin contents could be assayed immunochemically. At the same time the possible abnormalities of these globulins were suggested by their inability to

remove all the antibodies for the gamma globulin of normal serum. An example of this is illustrated in Fig. 2 by the myeloma serum which did not yield as much precipitate in the antigen excess zone as the normal serum.

TABLE V. COMPARISON OF IMMUNOCHEMICAL AND PAPER ELECTROPHORESIS ASSAYS

SAMPLE DESIGNATION	% OF TOTAL PROTEIN					
	ALBUMIN			GAMMA GLOBULIN		
	IMMUNO.			IMMUNO.		
	NEPH.	SPECT.	ELECT.	NEPH.	SPECT.	ELECT.
Wil —Normal adult	62.4		60.5	13.0	14.4	14.0
Ger —Normal adult	60.5		56.5	14.3	16.9	15.5
Bas —Normal adult	57.5		47.5	13.8	15.6	15.0
Sta —Normal adult	63.0	64.4	52.0	14.9	16.9	14.5
NHS —Normal adult		57.6	55.7	17.2	19.0	16.7
Atr —Normal child 5 yrs.	66.4	63.9	55.0	10.0	10.4	11.5
Ltr —Normal child 6 yrs.	66.8	65.2	58.0	10.1	11.7	11.0
Sma —Respiratory infection, infant 1 yr.		58.3	55.2		5.8	5.9
Hol —Treated agammaglobulinemia, child 3 yrs.		65.0	63.5	2.1	2.1	3.8
Wei —Allergic exzema, child 2 yrs.		48.0	43.8	19.3	23.2	20.7
Mar —Asthma, child 3 yrs.		61.4	61.7	11.0	11.9	12.2
She —Sprue	49	44.1	39.5	11.3	13.7	17.0
Kra —Nephrosis	13	14.1	8.5	11.7		12.0
Par —Nephrosis	18.2		16.0	12.0		13.0
Kla —Pylonephritis and cirrhosis	26.0	25.3	15.0	17.4	21.7	23.5
Ker —Rheumatoid arthritis, periarthritis	30.4	31.0	22.5	33.0		36.5
Mem —Polyarthritis	48.3	47.0	39.7		42.0	39.7
Koz —Biliary cirrhosis	34.4	33.2	25.5	46.7	46.0	51.5
Net —Bowel carcinoma	50.0	47.0	43.5		14.0	14.8
Uof —Active tuberculosis	54.0	56.0	42.4		17.0	15.4
Xis —Active tuberculosis	43.3	43.6	40.3		28.0	26.0
Raf —Lupus erythematosus	37.4	37.4	28.5		37.2	36.1
Bek —Lupus erythematosus (before cortisone)	31.1		25.0	38.8	48.0	49.0
Bek —Lupus erythematosus (after cortisone)	31.0		26.5	37.5	44.7	48.0
Zei —Multiple myeloma	32		28.0	19.5		25.5
Mei —Multiple myeloma—gamma type	15.8		18.0	60.4	62.0	62.0
Sul —Multiple myeloma—gamma type		10.0	8.9		87.0	80.7
Wal —Multiple myeloma—beta type		28.0	23.5		10.9	8.5
Opa —Multiple myeloma—$gamma_2$ type		22.6	13.2	56.6	58.1	51.5
Hun —Macroglobulinemia—$gamma_1$ type		18.0	17.0		?*	65.5

*The sample at 0.00025 ml. yielded maximal precipitation with anti-Fr II serum. This cross reaction was far too small, however, to permit accurate quantitation of the protein related to normal gamma globulin.

Comparison of Spectrophotometric and Nephelometric Methods in the Assay of Gamma Globulin.—Though interchangeable results were obtained in the albumin assay by either the spectrophotometric or nephelometric methods, this was not the case in the gamma globulin assay. As can be seen from Table V the spectrophotometric values for gamma globulin were somewhat larger than the nephelometric values. This difference could arise, in part, from the divergence of the reaction curve of human serum from that of Fr II. Such divergence, illustrated in Fig. 2 for the spectrophotometric method, began at lower antigen concentration and was larger in the nephelometric method. Since the reaction curves of various types of human serum followed that of normal human serum, it seemed worthwhile to construct the calibration curves for

both methods from a sample of normal human serum whose gamma globulin content had been assigned arbitrarily from the results of a previous assay. Table VI demonstrates that with such a standard the values then obtained by both methods were for practical purposes interchangeable.

TABLE VI. VARIATION IN GAMMA GLOBULIN LEVELS (% OF TOTAL PROTEIN) WITH DIFFERENT CALIBRATION CURVES

SAMPLE DESIGNATION*	NEPHELOMETRIC STD. CURVE			SPECTROPHOTOMETRIC STD. CURVE	
	FR II	NHS AT 14 MG./ML.	NHS AT 13 MG./ML.	FR II	NHS AT 13 MG./ML.
Wil —Normal adult (74.2)	12.8	13.8	12.5	15.4	14.0
Ger —Normal adult (72.0)	14.6	16.0	14.3	17.1	15.7
Bas —Normal adult (73.8)	14.2	15.2	13.6	16.3	14.6
Sta —Normal adult (78.0)	15.1	16.7	14.9	17.1	15.4
Atr —Normal child 5 yrs. (75.6)	9.9	10.5	9.2	11.6	9.9
Ltr —Normal child 6 yrs. (76.0)	9.9	10.6	9.2	12.3	10.6
She —Sprue (42.2)	11.3	11.8	10.6	15.3	13.7
Kla —Pylonephritis and cirrhosis (49.8)	17.6	18.4	16.6	23.6	21.0
Bek —Lupus erythematosus (74.5)	39.0	44.3	40.0	44.9	40.8
Bek —Lupus erythematosus (80.1)	37.4	42.3	38.6	43.5	39.7
Opa —Multiple myeloma (74.0)	56.6	60.5	54.0	54.4	50.4
Mar —Asthma, child 3 yrs. (82.3)	11.0	11.6	10.4	11.9	10.7
Wei —Allergic exzema, child 2 yrs. (75.5)	19.3	22.1	20.0	23.2	21.2
NHS—Normal adult (75.5)	18.5† 17.2‡ 15.9§	18.5	17.2	19.0	17.2

*Number in parentheses is milligram of total protein per milliliter of undilute sample.
†Used 0.001 ml. of NHS for analysis.
‡Used 0.002 ml. of NHS for analysis.
§Used 0.004 ml. of NHS for analysis.

It is of additional interest that these results were gathered with an antiserum (P-9) which had been produced to 200 mg. of Fr II and had evidenced a faint reaction for beta globulin in Oudin tests and in supernatant testing. On comparison with antiserum exhibiting complete specificity, P-9 yielded for a number of the serum samples slightly larger gamma globulin values by the spectrophotometric method but identical values by the nephelometric method when the standard curves were constructed from Fr II. Apparently, then, this data and that of Table VI suggest that the use of normal human serum of assigned gamma globulin content as the standard instead of Fr. II provides a means for neutralizing the effect of variable trace reactions encountered on comparing different batches of anti-Fr II serum. The need to neutralize such an effect is most likely to arise in the spectrophotometric assay in which trace reactions such as that of P-9 for beta globulin could develop as well as the predominant reaction for gamma globulin. At the much higher dilutions employed in the nephelometric assay in which the effective concentration of reactants is drastically reduced only the predominant reaction would appear to register.

The failure of minor antibody-antigen reactions to develop in the nephelometric method was also revealed in an experiment concerned with purifying an anti-Fr II serum (P-10). This antiserum, produced by the intravenous method,[3, 5] showed by Oudin tests a weak reaction for a non-gamma globulin component in addition to its potent gamma globulin reaction. Following the procedure of Jager[25] the unwanted antibodies were removed by absorption with the human serum globulin fraction which is soluble in 35 per cent Sat Am_2SO_4 but insoluble in 50 per cent Sat Am_2SO_4. Absorption with 125 mcg. of this globulin per milliliter of P-10 removed the weak reaction and still left a strong gamma globulin reaction. The antiserum both before and after the absorption was used to assay the amount of gamma globulin in the 35 to 50 per cent Sat Am_2SO_4 globulin fraction. By the spectrophotometric method a significant reduction in the gamma globulin assay took place after the absorption (from 7.24 per cent to 4.95 per cent), but by the nephelometric method both the unabsorbed and absorbed antisera yielded equal low values (4.23 per cent and 4.05 per cent respectively).

DISCUSSION

Human serum contains a large number of distinct proteins. A minimum of 16 species have been demonstrated by the technique of immunoelectrophoresis.[26] Many more are undoubtedly present. Most of these proteins migrate during electrophoresis as alpha and beta components and cannot as yet be individually assayed in human serum, nor can they be isolated in high purity. Those which can, such as orosomucoid and heat labile $alpha_2$ glycoprotein, have been successfully measured by immunochemical methods.[6, 27] The means for assaying a growing number of the other proteins may also be provided by the application of our particular immuno-nephelometric method in which only the predominant antibody-antigen reactions register. This should permit the use for assay purposes of antisera lacking complete specificity. However, the ratio of "unwanted" to "wanted" antibodies that can be tolerated in an antiserum still has to be established. On the whole, production of usable antisera can be expected due to the unique ability of the chicken to respond vigorously to a single antigen injection with a minimum of antibodies to contaminants.

The immunochemical method provides a powerful tool for clinical investigation of the plasma proteins. By this method changes in the plasma levels of individual small quantity as well as large quantity proteins can be accurately followed. The advantages of rapidity of analysis and simplicity of technique are also offered the investigator. In this regard the immunochemical technique is comparable to turbidimetric methods employing salting out or acid precipitation of proteins. While these latter methods suffer from their lack of specificity in the partitioning of protein mixtures and have sizable errors inherent to their use in that the physical character of the precipitates may vary from one sample to another, the immunochemical method is relatively free of such defects. First, each antiserum is specific for an individual plasma protein, and second, since assay is carried out in a large excess of antiserum the character of the precipitate is not affected by the nonantigen components of the sample.

The amount of protein contributed by the antiserum may be at least a hundred fold greater than that contributed by the sample and quantities of antiserum and salt (8 per cent) are constant in a series of determinations.

The demonstration by our data of a correspondence in the immunochemical and electrophoretic determinations of human albumin and gamma globulin helps establish the validity of our method. Basically, however, its validity must rest on the demonstration that each antiserum will effectively measure only a single antigenic type, for even in the assay of gamma globulin* there will not always be correspondence between the two methods. Slater[30] has reported examples in multiple myeloma sera of increased amounts of protein which electrophoretically were beta components but antigenically were related to normal gamma globulin. Conversely, examples of increased gamma-1 components bearing as antigens little or no relationship to normal gamma globulin have been cited.[31] The combination of both methods of assay in clinical study of the plasma proteins in disease obviously is more revealing than either method alone. In this regard our results with multiple myeloma sera suggest that a rapid immunochemical procedure can be worked out for the qualitative as well as quantitative examination of the proteins in pathologic serum samples by the reactions of these samples with the antisera produced to the normal plasma proteins. Failure of a sample to yield with an antiserum the same maximal reaction in the equivalence and antigen excess zones as normal serum would be indicative of an abnormal protein having replaced its normal counterpart.

As the immunochemical method is extended to the assay of a growing number of plasma proteins the selection of standards may pose certain problems. These can be described by our experiences with Fr II. This readily available fraction provided a good immunizing preparation for production of antisera specific to gamma globulin but yielded anomalous results when used as the standard in the gamma globulin assay. There was divergence of the reaction curves afforded by Fr II and by human serum resulting in a somewhat arbitrary assignment of gamma globulin value. In some cases the range of values for different dilutions of a sample was larger than the duplication error of the method. Certain of our data (unpublished) suggest that the aggregative changes that take place during alcohol fractionation can introduce artifacts into the specificity of the gamma globulin in Fr II. Changes in specificity and reactivity might well take place whenever prolonged fractionation is involved in the purification of a protein. Our proposed procedure for handling the gamma globulin assay (Table VI) could in this event have general application. By this procedure a pool of human serum or plasma of assigned values, determined by our best reagents,† for each of the proteins to be assayed would

*We have taken as our definition of gamma globulin that closely related antigenic material described by Williams and Grabar[28] which comprises in normal serum all of the gamma-2 area, most of the gamma-1 area, and in diminishing amounts some of the beta area. There is still conflict of opinion, however, as to whether this antigenic material extends beyond the gamma area[29] in normal serum.

†We are currently attempting to assign to a pool of lyophilized human plasma the most accurate immunochemical value for gamma globulin by employing as primary standards various preparations of gamma globulin, such as that isolated by starch electrophoresis.

constitute our routine standard. Then in sample assays comparative values of high accuracy could be expected. Furthermore, such a standard would help compensate for any trace contaminant reaction or undetermined cross reactions yielded by different batches of antisera produced to the same antigen.

SUMMARY

The feasibility of routine production in chickens of specific antihuman albumin and antihuman gamma globulin sera was demonstrated. Such antisera were successfully used as reagents for the assay of albumin and gamma globulin in human serum. For this purpose two rapid turbidimetric procedures of assay, applicable to the clinical laboratory, were worked out. It was found that in the high dilution of reactants employed in the nephelometric procedure, only predominant antibody-antigen reactions registered, thus increasing the effective specificity of the antisera. There was a close correspondence of the immunochemical and electrophoresis methods in the assay of albumin and gamma globulin. The possibility of measuring a broad spectrum of individual plasma proteins by application of our immunonephelometric method was discussed.

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